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PROFILING OF NUTRITIONAL TRAITS IN INDIGENOUS WHEAT CULTIVARS

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ABSTRACT

Farmers’ traditional crop varieties are the valuable source of diversity, nutritional heritage and genetically important traits. Wheat is an important cereal crop serving as a major source for energy and nutrients for millions throughout the world. Modern trend of promoting and cultivating high yielding variety has suppressed the existence of indigenous varieties and resulting in the loss of the same. Many traditional wheat cultivars have disappeared after the green revolution. By looking at the rich nutrient properties and needs of farmers’ crops varieties, many efforts are being under taken for their protection and promotion. Present study was performed to measure the nutrient levels in whole grain flour of thirteen different farmers’ wheat cultivars in comparison with check-released varieties. The average composition showed a significant variation among farmers’ and check wheat varieties; among various tested varieties, wheat cv. Mohit-gold reported with higher potassium (K) and magnesium (Mg) contents. While, Kudrat-7 contained higher fibre content whereas Rk-7 exhibited significantly higher content of carbohydrate and sugar. Overwhelming higher iron content was reported in Bansilocal, HZG-30 and Kudrat-7 cultivars.

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1 Introduction

India is a country of diversity where various crops and thousands of cultivars have been conserved and grown since ancient times. It has been reported that in India production of wheat, barley and jujube crop was started before about 9000 B.C. (Stein, 1998). Since then, there has been a revolutionary change in crop cultivation. Wheat is consumed as staple food source in almost every state of India where traditional and modern methods are used for the cultivation and consumption of wheat. Wheat flour and its value added products are primarily used as an energy food, but it is also known for its broad nutrient properties.

It is factual that if traditional cultivars are grown in an organic way, their nutrients can be used appropriately. During 1967-68 and 2003-04 when production of cereal crops increased two times, production of wheat crop was raised up by three times (Nagarajan, 2005). Due to this reason, green revolution in India is also known as ‘wheat revolution’. Wheat is the main crop, which remained benefited after the green revolution in India. It is ranked fourth among the top fifty agriculture commodities of the world and as per the report of United States Department of Agriculture, during 2015-16, India followed China in the leading wheat producing countries and shared 11.78 percent and 0.40 percent of worldwide wheat production and global wheat exports respectively (Anonymous, 2018).

In India, every year around sixty popular wheat varieties are cultivated, in which the top ten varieties account for sixty percent of the national demand of wheat (Nagarajan & Singh, 1997). Newly introduced and released varieties are trait specific and genetically uniform. These varieties are drastically used by most of the farmers for higher yield but these are also responsible for the loss of diversity of indigenous varieties and cultivars of wheat. India has rich experience and huge area with outstanding diversity for wheat cultivation (Nagarajan, 2005; Fernandes, 2017). Farmers’ cultivars are genetically diverse but after the green revolution, traditional varieties are rapidly disappearing resulting in loss of genetic diversity due to non-preference over hybrid varieties. For sustainable agriculture, affordable food production and food security, conservation of agrobiodiversity has been marked to play a vital role.

Major concern is associated with modern wheat crop varieties and its cultivation methods, which mainly focuses on higher yield, without concerning about the essential nutritional values and agrochemical toxicity issues (Reynolds et al., 1999; Garvin et al., 2006). Modern monoculture farming indirectly violates the balanced nutrition right of individual; it is also responsible for the increased incidences of micronutrient deficiencies (Yu & Tian, 2018). Such changes in major nutrition source coupled with sluggish lifestyle resulted enhance instances of cardiovascular disease, type two diabetes and cancer in many developing countries (Key et al., 2002; Musaiger & Al-Hazzaa, 2012). Due to this, various national and international initiatives are under way focusing diversity and nutrient density traits for variety improvement programs.

In present scenario, many farmers conserve & cultivate traditional varieties and improve the varieties through mass selection from the cultivars popular in the area. The cultivars either traditionally grown/ improved by the farmers or having a wild relative/land race or about which the farmers possess the common knowledge are known as Farmers’ plant varieties (Lushington, 2012). According to the recent figures of registered varieties in India under Protection of Plant Varieties and Farmers’ Rights (PPV & FR) Authority (2018), 42.45 percent of the crop varieties registered (under PPV & FR Act, 2001) were farmers’ varieties, where maximum 96.78 percent farmers’ varieties belongs to paddy only (till February 2018). For preserving crop genetic diversity and promoting the cultivation of traditional and farmers’ developed varieties, Government of India is working through public sectors organizations like Indian Council of Agricultural Research (ICAR) and its allied research institutes, Central and state Agricultural Universities, Non-governmental organizations as well as private sectors organizations. During 2015-17, the study was conducted by National Innovation Foundation, India, setup of Department of Science and technology, Government of India working as a national initiative to strengthen the grassroots technological innovations and outstanding traditional knowledge. The study mainly focussed on comparison of composition of thirteen farmers’ wheat varieties with two checks. The study intends to provide scientific evidence to the claims of the innovators and may act as a basis for inclusion of these nutrient rich cultivars in the national nutrition supplement programs.

2 Materials & Methods:

2.1 Experimental design

For obtaining the wheat flour samples for the analysis a randomized block design containing fifteen wheat cultivars (13 farmers’ and 2 checks) with three replications was sown in Gandhinagar (23.376064° N, 72.719488° E), Gujarat, during 2015-2017. This study included fifteen potentially cultivated wheat varieties from different agro climatic wheat cultivation zone viz. twelve farmers’ cultivars from the database of National Innovation Foundation - India, one from farmers’ check durum variety Bansi-local and two check varieties (GW-496 and HD-2967) most widely cultivated in Gujarat and North East & North West Zone respectively (Table 1).
2.2 Estimation of nutrient and mineral content in farmers’ wheat varieties

2.2.1 Preparation of sample

The primary step before milling is cleaning of grains, which was carried out manually to eliminate foreign seeds, soil particles and broken kernels. Each sample of wheat varieties were allowed to dry for 48 hours and subsequently underwent milling at laboratory by using Multipurpose Pulveriser Machine (Sieve size -1), Savalia Electricals, Ahmedabad, Gujarat, India. The flour samples of each variety were sealed in air-tight containers and placed at 18 °C in a deep freezer for storage. The flour samples were thawed at room temperature prior further analysis. For this study, the wheat flour were analysed for the content of carbohydrate, protein, crude fibre, total soluble sugar, moisture, total fat, wet gluten, dry gluten and essential minerals such as iron (Fe), calcium (Ca), magnesium (Mg) and potassium (K).

2.2.2 Estimation of major nutrient components

Total carbohydrate content in the samples was assayed by the Anthrone method by taking glucose as the standard, whereas total soluble sugar was estimated by following the method of Dubois (Dubois et al., 1951; Yemm & Wils, 1954). Moisture and ash content of the flour was determined by standard AACC procedure (AACC International, 2000). Total protein content in the samples was determined by the modified Lowry method (Lowry et al., 1951; Hartree, 1972). Total fat and crude fibre content of the sample was extracted and estimated by Nielsen and standard AOAC methods (AOAC, 1990; Nielsen, 1994), whereas the wet gluten and dry gluten content were analysed by standard method of gluten estimation (ISO 2170-1980, 1980; Kaushik et al. 2015).

2.2.3 Estimation of essential minerals

The essential mineral contents in the fine wheat flour samples were determined by methods prescribed in AOAC (2000). Absorbance of standard solution as well as sample solution was taken by an atomic absorption spectrophotometer (ICP MS Elan 9000. Perkin Elmer., USA).

2.3 Statistical analysis

Data generated from the assay of the wheat samples was compared by the analysis of variance (ANOVA) (0.05 level), Duncan multiple range test (at P<0.05 significance level) and Pearson correlation (0.05 levels) coefficients with the help of GraphPad Prism (version 5.01).

3 Results

Nutrient contents of thirteen farmers’ and two checks wheat varieties were calculated on different basis to allow comparison with literature data. ANOVA showed that there were significant differences (P < 0.05) in the composition of wheat accessions (Verma & Shrivastav, 2017).
Profiling of nutritional traits in indigenous wheat cultivars

The carbohydrate, protein, moisture, wet gluten and dry gluten in seven wheat varieties. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference according to Duncan multiple range test at $P<0.05$ significance level.

Figure 1 The carbohydrate, protein, moisture, wet gluten and dry gluten in eight wheat varieties. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference according to Duncan multiple range test at $P<0.05$ significance level.

Figure 2 The carbohydrate, protein, moisture, wet gluten and dry gluten in seven wheat varieties. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference according to Duncan multiple range test at $P<0.05$ significance level.

carbohydrate except *Bansi-local* which showed 59.2 per cent of carbohydrate. Significantly higher carbohydrate content was found in Rk-2 and Rk-7 varieties with 73.9 and 75.0 percent respectively. Commercially cultivated varieties *HD-2967* (68.5 %) showed highest carbohydrate composition as compared to *GW-496* (64.5 %) (Figure 1 & 2).

Among all varieties, maximum protein content was found in *HD-2967* variety, which was comparable to *BLK-balaji* (13.2 %) and *Rajyog* wheat (13.1 %). Check wheat variety *GW-496* exhibited protein content as 13.1 per cent, which was found comparable with *Rk-Shital* (12.4 %), *Kudrat-7* (12.5 %), *AR-64* (12.6 %), *Bansi-local* (12.9 %), *Rk-7* (12.9 %), *Kudrat-17* (12.9 %) and *Rajyog* (13.1 %). Significant lower protein content was found in *Kudrat-9* (11.9 %) and *Rk-2* (12.0 %) wheat varieties (Figure 1 & 2); however, the values were higher as compared to average value of protein content.

### 3.2 Moisture and gluten content

The moisture percent of wheat varieties varied between 7.20 - 10.20 per cent (Figure 1 & 2). The maximum moisture percent was exhibited in *Kudrat-9* (10.2 %), whereas significantly lower moisture was in *AR-64* wheat flour sample (7.2 %). Among the tested varieties, significantly higher wet gluten was exhibited by check variety *HD-2967* (41 %) followed by *Bansi-local* (39 %) and *GW-496* (38 %), however maximum dry gluten percent was found in Rk-2 (13.91 %) followed by Rk-7 (13.14 %), Rk-4 (13.14 %) and *Bansi-local* (11.66 %). Significantly lower wet gluten was found in *Kudrat-17* (30 %) followed by Rk-4...
(31 %), whereas significantly lower dry glute percentage was found in GW-496 (6.32 %) followed by Kudrat-9 (6.62 %) and Rk-Shital (6.89 %) (Figure 1 & 2).

3.3 Total ash and fat percent

Highest ash content (2.07 %), followed by Mohit-gold (2.03 %), whereas AR-64 Farmland’s wheat variety Rk-7 showed exhibited lowest ash percent (1.45 %) (Figure 3 & 4). Among the check varieties, GW-496 showed higher ash content (1.98 %), whereas HD-2967 showed lower ash content (1.83 %). Fat values were found significantly different among wheat cultivars, farmer’s variety AR-64 showed 3.00 per cent fat content whereas Bansí-local and Rajyog exhibited 2.8 per cent fat value, which was higher as compared to HD-2967 (2.70 %) and significantly higher as compared to fat content of GW-496 (2.6 %), whereas Kudrat-9 exhibited the lowest fat content (1.60 %) (Figure 3 & 4).

3.4 Total sugar and fibre content

Total sugar content of farmers’ wheat varieties and check varieties is mentioned in figure 3 & 4. Maximum sugar content was exhibited by Rk-7 (4.8 %) and Rk-Shital varieties (4.2 %), whereas minimum total sugar content was found in Kudrat-17 (1.40 %) followed by GW-496 (1.5 %). The fibre percent of all the wheat accessions were found above 1.0 per cent. Among the tested varieties, Kudrat-7 exhibited the significantly higher fibre percentage (2.90 %), followed by Kudrat-17 (2.70 %), HZG-30 (2.60 %) and Kudrat-9 (2.40 %), whereas Bansí-local contained the lowest fibre (1.3 %) followed by Rajyog wheat variety (1.4 %) (Figure 3 & 4).
3.5 Essential minerals content

Microelement iron (Fe) was found as the variant mineral component in farmers’ and check varieties of wheat with a range of 35 - 175 mg/kg. The abundance of iron was found in all the cultivars, which can be due to the soil and environmental conditions also, it is a subject of further investigation. Among all the varieties, remarkably high Fe was exhibited in Bansi-local (175 mg/kg) followed by HZG-30 (95 mg/kg), Kudrat-7 (85 mg/kg) and Kudrat-17 (70 mg/kg) (Figure 5). Range of calcium (Ca) and magnesium (Mg) was found as 325-735 mg/kg (Figure 5) and 1105-1970 mg/kg (Figure 6) respectively. Significantly higher

Figure 5 The iron (Fe) and calcium (Ca) content in farmers’ wheat varieties. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference according to Duncan multiple range test at P<0.05 significance level.

Figure 6 The magnesium (Mg) and potassium (K) content in farmers’ wheat varieties. Data are means±standard error of three replicates (n=3). Bars followed by different letters show significant difference according to Duncan multiple range test at P<0.05 significance level.
Ca and Mg content was found in Rajyog (735 and 1970 mg/kg respectively) followed by Kudrat-9 having 725 mg/kg as Ca content and Bans-local with 1665 mg/kg as Mg content, however minimum Ca content was found in Rk-2 (325 mg/kg) followed by Rk-7 (335 mg/kg). Significantly, lower Mg content was found in Kudrat-17 (1105 mg/kg), Kudrat-9 (1160 mg/kg) and GW-496 (1320 mg/kg) (Figure 5, 6). Average Ca and Mg content were found as 447 and 1468.7 mg/kg respectively.

Average potassium (K) content in our study was found as 5133.3 mg/kg and the range was 4000-6000 mg/kg (Figure 6). Maximum K content was found in Bans-local and Mohit-gold (6000 mg/kg) followed by Rajyog, HZG-30, BLK-balaji, Rk-Shtial and HD-2967 (5500 mg/kg) whereas minimum K content was recorded in Rk-2 (4000 mg/kg) followed by Kudrat-9, AR-64 and GW-496 (4500 mg/kg).

The correlation among the nutrient components and mineral compositions in farmers’ and check wheat cultivars (Table 2) exhibited that fat percent was in moderate positive correlation with Mg content (r = 0.583, P = 0.023) and negative relationship with moisture percent (r = -0.759**, P = 0.001) at 0.01 level (Table 2).

4 Discussion and Conclusion

Present study revealed that farmers’ wheat cultivars contain higher nutrient & mineral contents and may be considered as promising genetic source for improving the nutrient values. Rajyog variety was found with higher protein, calcium, magnesium and possessed medium iron content. Bans-local was found with higher Iron, magnesium, potassium, protein content and medium calcium content. Kudrat-17 was found with lower wet gluten content and medium to higher protein, fiber as well as iron content. HZG-30 was found with higher iron, Potassium, fiber content and medium calcium, wet gluten content. Mohit-gold was found with higher potassium content and medium to higher carbohydrate and fiber contents. BLK-balaji was found with higher potassium and protein content and medium to higher calcium and carbohydrate content. Check varieties HD-2967 and GW-496 contained highest protein content, which was comparable with BLK-balaji variety.

4.1 Carbohydrates content

The farmers’ and released varieties were found comparable with the average carbohydrate values in wheat, which is 59.20 to 75.00 per cent (Souci et al., 2008; Belitz et al., 2009). Carbohydrates are an important energy source for human body. Wheat flour is having complex carbohydrates that also characterize the presence of calories level in it. Variation in carbohydrate compositions in wheat occurs mainly due to various factors like genotypes and cultivation conditions (Stone & Morell, 2009). In earlier studies, it has reported that the carbohydrate range was 65 to 75% in American and Korean traditional wheat varieties (Stone & Morell, 2009; Choi et al., 2016), whereas among 31 different Korean wheat cultivars, minimum carbohydrate content was found in Shinmichal-1 variety (67.87 %) and highest carbohydrate content was 74.6 % in Jeokjoong variety (Choi et al., 2016). These results are in agreement with the findings of present study, which revealed that the farmers’ wheat cultivars are diverse in carbohydrate content.

4.2 Protein content

The protein content in farmers’ and check wheat varieties were found significantly higher i.e. more than 11.3 per cent (Triboi et al., 2003; Koehler & Wieser, 2013). Protein may vary from 6 to more than 20 per cent in wheat cultivars depends on genotype and growing conditions (Triboi et al., 2003). Protein content is an important factor for quality; value added processing and economical value of wheat flour. Protein percent in wheat depends on variety and cultivation conditions (Triboi et al., 2003; Zhao et al., 2009). According to Cooper (2005) value of protein ranges from 10 to 15 per cent, in the context, this study corresponds to the previous study. Present study also revealed that in terms of protein content farmers’ developed wheat varieties are rich which is alike to check released varieties under Gandhinagar condition.

4.3 Moisture content

The moisture percent of wheat varieties were found in the range of adequate level (>11.5%) for longer storage and better avoidance of insect-pest spoilage (Ahmed et al., 2016). In a previous study comprised flour samples of seven traditional wheat varieties i.e. Inqulah-91, Bhakkar-2002, AS-2002, Shafaq-2006, Sehar-2006, Auqab-2000 and GA-2002 belongs of Punjab, Pakistan, it was found that minimum moisture was 9.64 per cent (Bhakkar-2002) and 9.79 % (GA-2002) as maximum moisture content (Saifdar et al., 2009). The moisture content is essential to maintain the final product quality. Present study revealed that the moisture level in wheat flour sample for both farmers’ developed and check varieties was found in safe range which suggests its suitability for market and domestic usage.

4.4 Gluten content

During the study, the wet and dry gluten contents ranged from 30.00 to 41.00 per cent and 6.32 to 13.91 per cent, respectively. As gluten is responsible for the elasticity and extensibility characteristics of flour dough, testing of wet gluten provides the information regarding quantity and help in estimation of the quality of gluten in wheat or flour samples. Wet gluten also
Table 2 Pearson correlation coefficients among nutrient composition values in farmers’ and released check wheat varieties

<table>
<thead>
<tr>
<th></th>
<th>Dry gluten %</th>
<th>Total ash %</th>
<th>Wet gluten %</th>
<th>Moisture %</th>
<th>Total fiber %</th>
<th>Total sugar %</th>
<th>Fat %</th>
<th>Protein %</th>
<th>Carbohydrate %</th>
<th>K (mg/kg)</th>
<th>Mg (mg/kg)</th>
<th>Ca (mg/kg)</th>
<th>Fe (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry gluten %</td>
<td>0.182 (0.515)</td>
<td>-0.428 (0.111)</td>
<td>-0.169 (0.548)</td>
<td>-0.161 (0.568)</td>
<td>0.133 (0.635)</td>
<td>0.179 (0.524)</td>
<td>-0.232 (0.406)</td>
<td>0.480 (0.070)</td>
<td>-0.114 (0.686)</td>
<td>0.157 (0.576)</td>
<td>-0.464 (0.081)</td>
<td>0.248 (0.373)</td>
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<tr>
<td>Total Ash %</td>
<td>0.015 (0.958)</td>
<td>0.517 (0.049)</td>
<td>0.142 (0.614)</td>
<td>0.048 (0.864)</td>
<td>-0.381 (0.161)</td>
<td>-0.064 (0.821)</td>
<td>0.374 (0.170)</td>
<td>0.240 (0.389)</td>
<td>0.017 (0.951)</td>
<td>0.014 (0.960)</td>
<td>-0.114 (0.685)</td>
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<tr>
<td>Wet gluten %</td>
<td>0.150 (0.594)</td>
<td>-0.325 (0.237)</td>
<td>-0.233 (0.403)</td>
<td>0.134 (0.634)</td>
<td>0.433 (0.107)</td>
<td>-0.053 (0.852)</td>
<td>0.400 (0.140)</td>
<td>0.137 (0.628)</td>
<td>0.183 (0.513)</td>
<td>0.320 (0.245)</td>
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<tr>
<td>Moisture %</td>
<td>-0.249 (0.370)</td>
<td>-0.047 (0.868)</td>
<td>-0.043 (0.878)</td>
<td>0.207 (0.459)</td>
<td>-0.220 (0.432)</td>
<td>-0.378 (0.164)</td>
<td>-0.064 (0.820)</td>
<td>-0.279 (0.314)</td>
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<td>Total fiber %</td>
<td>0.016 (0.140)</td>
<td>-0.399 (0.001)</td>
<td>-0.759 (0.188)</td>
<td>-0.360 (0.518)</td>
<td>0.181 (0.966)</td>
<td>0.012 (0.060)</td>
<td>0.020 (0.309)</td>
<td>0.084 (0.765)</td>
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<tr>
<td>Total sugar %</td>
<td>-0.043 (0.879)</td>
<td>0.006 (0.798)</td>
<td>0.305 (0.269)</td>
<td>0.067 (0.814)</td>
<td>0.310 (0.260)</td>
<td>0.320 (0.245)</td>
<td>0.064 (0.250)</td>
<td>0.317 (0.175)</td>
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<td>Fat %</td>
<td>0.502 (0.057)</td>
<td>-0.002 (0.993)</td>
<td>0.058 (0.837)</td>
<td>0.583 (0.023)</td>
<td>-0.167 (0.551)</td>
<td>0.353 (0.197)</td>
<td>0.378 (0.117)</td>
<td>0.139 (0.708)</td>
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<td>Protein %</td>
<td>0.183 (0.515)</td>
<td>0.348 (0.203)</td>
<td>0.329 (0.231)</td>
<td>-0.038 (0.893)</td>
<td>0.080 (0.778)</td>
<td>0.080 (0.778)</td>
<td>0.320 (0.231)</td>
<td>0.139 (0.708)</td>
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<tr>
<td>Carbohydrate %</td>
<td>-0.274 (0.323)</td>
<td>0.095 (0.735)</td>
<td>-0.328 (0.232)</td>
<td>0.347 (0.206)</td>
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<td>K (mg/kg)</td>
<td>0.365 (0.180)</td>
<td>0.149 (0.597)</td>
<td>0.348 (0.204)</td>
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<td>Mg (mg/kg)</td>
<td>0.175 (0.532)</td>
<td>0.112 (0.691)</td>
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<tr>
<td>Ca (mg/kg)</td>
<td>-0.064 (0.822)</td>
<td>0.002 (0.993)</td>
<td>-0.064 (0.822)</td>
<td>0.002 (0.993)</td>
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<tr>
<td>Fe (mg/kg)</td>
<td>0.365 (0.180)</td>
<td>0.149 (0.597)</td>
<td>0.348 (0.204)</td>
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reflects the protein content of flour. During an earlier study in Haryana, India comprised five different wheat varieties viz. C-306, H-977, HW-2004, PBW-550 & WH-542, it was found that C-306 contained maximum (11.7 %) dry gluten whereas minimum (7.7 %) dry gluten was found in HW-2004 (Dangi & Khatkar, 2017). In Madhya Pradesh, among four wheat cultivars KW-11, PBW-343, C-306 and Raj-3765 maximum wet gluten was exhibited in C-306 (36.54 %) whereas minimum wet gluten was found in PBW-343 (30.28 %) (Kaushik et al., 2015). These results are in agreement with the findings of present study, which revealed that the farmers’ wheat varieties had a wide range of wet and dry gluten content.

### 4.5 Ash content

The ash content has nutritional significance in wheat flour that represents the presence of mineral but excess ash also has bad impacts like undesirable darkening in dough and food products. In present study, ash percent was found significantly different in farmers’ wheat varieties in the range of 1.45-2.07 per cent. In an earlier study conducted at Haryana and Punjab, which had included nine Indian wheat varieties shown a wide range of ash content from 1.14 to 2.15 per cent, WH-1021 variety had the lowest ash content, whereas WH-157 had the highest ash content (Panghal et al., 2017).

### 4.6 Fat content:

Fat is an important component of our diet, which is used as a concentrated source of energy, supplies nine calories per gram. Fat from wheat is associated with essential oils, choline, and vitamin E. In an earlier study conducted with twelve different released varieties viz. WH-283, WHD-943, PBW-590, WH-1080, WH-896, WH-711, PBW-550, DBW-17, WH-542, WH-1025, WH-147 and PBW-343 from North India, the range of fat percentage was found as 2.62 - 3.48. Maximum fat was exhibited by PBW-
590 whereas minimum fat percentage was found in WH-1025 (Punia et al., 2017). The results are in agreement with the findings of present study, which revealed that the farmers’ wheat varieties contained fat in the range of 1.6 to 3.0 per cent.

4.7 Sugar content

Starch and sugars play important role for plant growth as well as for human consumption. Starch and sugars supply 3.75 calories per gram. Sugars and starch both are regarded as available because they are easily digested and converted to energy. Present study confirmed the wider range of total sugar content in farmers’ wheat cultivar (1.3 to 4.8 %). Among earlier studied Indian wheat varieties viz, RSP-566, RSP-561, PBW-396, HD-2687, C-306, PBW-175, RSP-81, PBW-550, DBW-17 and WH-542, maximum total sugar content was reported in PBW-175 (5.8 %), whereas minimum value of total sugar content was found in HD-2687 (3.01 %) (Mallick et al., 2013).

4.8 Fibre content

Whole grain wheat flour contains both soluble and insoluble form of fibres, which are derived from wheat bran. Soluble and insoluble form of fibres play significant role in nutrition and digestion process. The fibre content of the wheat cultivars were found in the range of 1.3 to 2.9 per cent. The results of present study was found in agreement with a previous study where six different wheat varieties from four countries viz. Agil, Mulan, Zentos (Germany); Kovas DS (Lithuania); Mariboss (Denmark) and Rigi (Switzerland), maximum crude fiber content was found as 2.19 per cent in Mariboss (Denmark), whereas minimum crude fiber was found as 1.63 % in Rigi (Switzerland) (Alijosius et al., 2016).

4.9 Mineral content

More than ten percent (>0.8 billion) people around the globe are malnourished whereas around twenty-five percent (>2 billion) people are prone to single or multiple micronutrient deficiencies. Due to insufficient micronutrient content in food, around 50 percent of people of the world were at the risk of calcium (Ca) deficiency during 2011 (Kumsa et al., 2015). Iron deficiency is an essential concern in India where maximum number of women are affected with anaemia, followed by China, Pakistan, Nigeria and Indonesia (Rai et al., 2018). The farmers’ varieties may provide a solution to solving the problems of acute nutrient deficiency.

In our study farmers’ cultivar were found rich in iron content as compared to the checks and earlier reported wheat varieties (Eagling et al. 2014; Alijosius et al., 2016; Panghal et al., 2017; Punia et al., 2017). Results are in agreement with the earlier report, which suggested that the iron content in wheat varieties found in wide variation (Qureshi et al., 2002; Oury et al., 2006; Zhao et al., 2009) and study which revealed wide variation in the iron content in wheat cultivars as compared to modern wheat (Cakmak et al., 2000; Monasterio & Graham 2000). However, richness of iron was exhibited in most of the cultivars of our investigation, which may be due to soil and environmental factors and may be considered for further investigation. Ca and Mg contents in farmers’ wheat varieties were found in agreement with earlier study where sixty-three wheat cultivars (56 historical, 7 modern) were analysed at Washington, USA and found Ca and Mg content in the range of 228- 568 mg/kg and 1145 - 1723 mg/kg respectively (Murphy et al., 2008). During their study, average Ca and Mg content was 417.9 and 1388 mg/kg respectively (Murphy et al., 2008), however in our study it was 447.00 and 1468.7 mg/kg respectively. The maximum K content was found in Bansi-local and Mohit-gold as 6000 mg/kg however in as similar study conducted in Sweden, where 321 organically cultivated winter and spring wheat cultivars were analysed and found maximum K content in primitive wheat cultivars as 4670 mg/kg (Hussain et al., 2010). In present study only fat percent showed moderate positive correlation with Mg content and negative relationship with moisture percent however in a similar study wheat protein was negatively correlated with carbohydrate (r= -0.93) (Khan et al., 1987).

The farmers’ wheat cultivars with potential nutritional values may be identified disseminated and considered for nutritional oriented breeding program to develop new varieties and can also be incorporated in the nutrition supplement programs of the government to ensure nutrient security among the vulnerable population of women and children across the country.

Acknowledgement

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Conflict of interest

All the authors declare that there is no conflict of interest.

References


ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF LOW MOLECULAR WEIGHT PEPTIDES FROM NONPRIMED AND HALOPRIMED SEEDLINGS of Vigna mungo L. AND Cajanus cajan L. AND THEIR IMPACT ON PHYSIOLOGICAL ASPECTS UNDER NaCl EXPOSURE

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ABSTRACT

Plants confront an array of environmental stresses by tightly regulating their signalling pathways involving low molecular weight peptide synthesis. In the conducted study, initial growth parameters and ion contents were measured from salt stressed and haloprimed seedlings of blackgram (Vigna mungo L.) and pigeonpea (Cajanus cajan L.). Seedlings raised from nonprimed and haloprimed seeds were grown in hydroponic solution supplemented with desired concentration of NaCl for 3 weeks under controlled physiological conditions. Attempts were made to isolate low molecular weight peptide(s) from non-stressed, NaCl stressed and haloprimed seedlings of blackgram and pigeonpea, to quantify their variations and detect their molecular weight using HPLC and MALDI-TOF analysis respectively. Free radical scavenging activities of the peptides were studied under NaCl exposure. Subsequent bioassays were performed to determine the effect of these peptides on their respective physiological parameters. Peptide abundance was maximum in control seedlings of both the cultivars, which under NaCl stress became scanty. CD spectroscopic analysis confirmed reduced secondary conformations and more unordered peptides under NaCl stress. Haloprimed seedlings recovered such adversities to variable extents, resulting in improved germination and growth of test seedlings.

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KEYWORDS  
CD spectroscopy  
Halopriming  
HPLC analysis  
Low molecular weight peptides  
MALDI-TOF  
Salt stress
1 Introduction

Salt stress is an increasing environmental challenge that limits plant productivity in arid and semi-arid regions of the world (Hussain et al., 2009; Etesami & Beattie, 2018). To confront under salinity, plants alter their physiological and biochemical mechanisms to withstand resulting stress (Parida & Das, 2005). Biologically active low molecular weight (LMW) peptides play regulatory role in determining several physio-biochemical processes in plants under abiotically stressed conditions. Some of them help in amplification of signals (Lindsey et al., 2002), some act as regulators of signalling events and cell-to-cell communication in plants (Murphy et al., 2012; Albert, 2013; Czyzewicz et al., 2013; Matsubayashi, 2014; Oh et al., 2018), whereas some play essential role in nitrogen fixation (Mylona et al., 1995; Muñoz-Garcia & Ares, 2016). Small peptides play critical roles in cell proliferation, differentiation and mediate biotic and abiotic stress stimulation with metabolic intermediates (Mandal et al., 2008). Small signalling cysteine rich peptides have also been reported to play essential roles in stomatal patterning and density, symbiosis and a wide range of reproductive events viz., pollen tube formation, guidance and burst, activation of gametes and seed development (Hara et al., 2007; Sugano et al., 2010; Marót et al., 2015; Bircheneder & DreselHaus, 2016). Few of these have been isolated and characterized in details. Systemin is probably the first sequenced plant peptide hormone (18 amino acid in length) which was isolated from Solanaceae species. Till now, peptides have been isolated and characterized in plants like bitter melon (Leung et al., 1987), broad bean (Zhang & Lewis 1997), wheat (Ghosh et al., 2010) and mulberry (Jha et al., 2014). However, it has not been isolated from pulses like blackgram (V. mungo) and pigeon pea (C. cajan) exposed to high NaCl concentrations, to the best of our knowledge. Variations in levels of oxidative stress markers, activities of enzymatic and non-enzymatic antioxidants in V. mungo and C. cajan has been studied under salinity (Chatterjee et al., 2018) but, the variations in levels of the isolated LMW peptides from the same stands undone, so far as halopriming is involved. These pulses have been selected to work with since they are essential pulses often consumed as the cheapest source of protein. The prime purpose of the study lies not only in isolation and partial characterization of LMW peptides (having molecular weight between 0.3-5.0 kDa) induced by NaCl stress but also detection of antioxidative properties and conformational changes of these peptides in the chosen materials. The study also detects the efficacy of these LMW peptides isolated from haloprimed seedlings that favoured stress alleviation imparting better seedling performance. The worth of the study lies in the fact that since pulse production is extensively hampered in the saline regions, adopting cost effective, farmer friendly and environment friendly halopriming technique may help in improving pulse vigour and stand due to endogenous production of LMW peptides in abundance that could improve legume germination and growth of test cultivars under saline conditions.

2 Materials and methods:

Fresh and viable seeds of V. mungo cv. Sulata and C. cajan cv. rabi were sourced from Pulse and Oilseed Research Institute, Behrampore, West Bengal. Seeds surface sterilized with 5% sodium hypochlorite solution for 15 minutes were divided into two batches. First batch was directly plated onto sterilized glass plates containing moistened blotting papers followed by their insertion into transparent plastic packets containing hydropnic solution (Widodo et al., 2009) which constituted the control set. For seeds to grow under salt stress, hydropnic solution (pH 6.8) was supplemented with 150mM NaCl. This constituted the nonprimed sets. The second batch of seeds were allowed to soak in sublethal dose of NaCl (50mM NaCl) for 2 hours for the purpose of halopriming, prior to their germination in hydropnic solution containing 150mM NaCl. The set ups were exposed to 16 hours of photoperiod at 27-30 °C and 200μmolm-2s-1 photon irradiance (Biswas et al., 2018). Previous report (Chatterjee et al., 2018) revealed that at 150mM NaCl, significant effect of salt toxicity was recorded in the test cultivars. Therefore, this dose was chosen for conducting the present study. After 21 days of growth, seedlings of each cultivar were harvested for peptide isolation. Extractions were carried out in cold room at 4°C.

2.1 Determination of morphological parameters

After 21 days of growth, 10 seedlings were randomly harvested from control sets, salt stressed sets (150mM NaCl) and haloprimed sets (50mM NaCl primed followed by their exposure to 150mM NaCl) of V. mungo and C. cajan seedlings for determination of salt induced damage in root and shoot length (Saha et al., 2010).

2.2 Estimation of Na⁺, K⁺ and Cl⁻ contents

1g of dried root and shoot samples from each set were utilized for Na⁺ and K⁺ ion estimation (Saha et al., 2012). Samples were placed inside muffle furnace at 450 °C for 24 hours in weighed porcelain crucibles to generate ash. The collected ashes were dissolved in 10 ml of 2 (N) HCl. The ash- acid extracts were filtered through Whatman filter paper (No. 42) into a 50ml volumetric flask. The volume was then made upto 50ml with 2 (N) HCl. This filtrate was used for Na⁺ and K⁺ ion estimation using a Systronics Flame photometer Model No. 130.

Chloride (Cl⁻) contents were estimated from fresh shoot and root samples by titration (Sheen & Kahler 1938). 1g root and shoot samples from each treatment was used for Cl⁻ estimation. Samples were crushed in 10ml of Milli-Q water to which a pinch of
charcoal was added and left undisturbed for 10 mins. Plant extracts were made charcoal free by repeated filtration and centrifugation. Cl contents were estimated from filtrates by silver nitrate titration method.

Data obtained for the two cations (Na⁺, K⁺) were expressed in mg g⁻¹ dry weight and Cl⁻ contents were expressed in mg g⁻¹ fresh weight.

2.3 Extraction and isolation of LMW peptides

For isolation and purification of LMW peptides, freshly harvested samples were pulverized in mortar and pestle using liquid nitrogen followed by its extraction in pre-chilled autoclaved distilled water. Extracts were centrifuged at 10,000 rpm with protease inhibitor PMSF for half an hour to remove interfering materials. Collected supernatant was again centrifuged to purify it.Repeated ether washes of the extracts helped to remove fats, lipids and oils (Jha et al., 2016). The extracts were thereafter preserved at -20°C for future experiments.

2.4 Ion exchange Chromatography

The peptide extracts were allowed to pass through cation and anion exchange resin columns (Dowex 50 and Dowex 1, Sigma Chemical Co., USA) (Azzoni et al., 2005). 3N ammonia and 1N HCl was used for cation and anion exchange column respectively during peptide elution procedure. Extracted solution was made ammonia and HCl free with a Lyophilizer (Lyolab BII). Thus, the extracts were freeze dried to smaller volumes.

2.5 Ultrafiltration

Lyophilized peptide extracts obtained from control seedlings, NaCl treated seedlings and haloprimed seedlings were ultrafiltered through millipore stirred cell fitted with 5 kDa (YM 5) and 0.3 (YC 03) ultrafilter membrane (Amicon made). Thus, the filtrate collected had peptides having molecular weight between 0.3-5.0kDa (Zehadi et al., 2015). During ultrafiltration, maximum pressure of 1.5kg/cm² was maintained. The process was carried out thrice and the ultrafiltered samples were lyophilized and dissolved in 10ml distilled water (for each set) and cryopreserved.

2.6 Purification and analysis of peptides through HPLC analysis

High performance liquid chromatography (HPLC), Waters™ was performed with the extracted peptides. C18 column was used in 50% methanol (running solvent) fitted with 515 HPLC pump. A run time of 20 minutes and absorbance at 250nm was recorded. Column length was of 3.9mm X 150mm, injection volume 20µl, flow rate of 0.5-1.0ml/min (Jha et al., 2014) with pump pressure 4000psi was maintained. Peptide(s) obtained at retention time around 5min was purified, concentrated and kept at -20 ºC. The obtained peak was isolated and re-injected into column to check its repetitive occurrence. Thus, these peaks were concentrated and collected to study their effect on various physiological aspects.

2.7 MALDI-TOF analysis

Extracts purified through HPLC analysis were subjected to MALDI-TOF mass spectra measurements carried out on Ultraflextreme MALDI-TOF/TOF spectrometer, Bruker Corporation, Massachusetts, US, equipped with nitrogen laser wavelength. Dihydroxybenzoic acid (DHBA) was used as matrix. 30 mg mL⁻¹ DHBA prepared in water: acetonitrile (1:1). Individual peptide extracts were mixed thoroughly in 1µl of DHBA matrix solution and applied on sample plate in volume of 1µl. Mixtures on sample plate were dried in an air stream followed by plate insertion into instrument for acquiring their respective spectra (Castañeda-Ovando et al., 2012).

2.8 Circular Dichroism Spectroscopy

Conformational changes in secondary structure of the isolated peptides was determined using CD spectropolarimeter (Model J-715, Jasco, Tokyo, Japan) at 25°C equipped with a temperature control unit using a 0.1cm path length quartz cell (Senthilkumar et al., 2008). Spectral scans were undertaken within wavelength range of 280-195nm. The percentages of secondary structures were deconvoluted using BESTSEL program.

2.9 Bioassays

Isolated LMW peptides from 21days old V. mungo and C. cajan seedlings were used to test the following physiological traits:

2.9.1 Germination test

Surface sterilized seeds each of V. mungo and C. cajan were plated in petriishes to test their germination ability under salt exposure in presence of the peptides isolated respectively from their control seedlings, 150mM NaCl treated seedlings and 150mM NaCl treated haloprimed seedlings. Three replicates were made for each set of seeds. The set ups were kept in plant growth chamber at 21 °C, 68% humidity for one week under controlled condition of 16 hours of photoperiod followed by 8 hours of darkness.

2.9.2 Stomatal bioassays

Peels were made from abaxial surface of Colocasia esculenta L. leaves at 11am. Initially peels were placed in water for two hours to prevent shock. Thereafter, peels were soaked in peptides isolated from control sets, NaCl treated sets and haloprimed sets
of V. mungo and C. cajan for 60 mins and mounted on glass slides to study the effect of LMW peptides on opening and closure of stomatal aperture.

### 2.9.3 DPPH radical scavenging assay

Free radical scavenging activities of the isolated peptides was assayed (Chrysargyris et al., 2016). Reaction mixture contained 1ml of 0.3mM DPPH (1,1-diphenyl-2-picrylhydrazyl) and 0.02ml of aqueous peptide extracts. The reaction mixtures were vortexed and incubated in dark for 30 minutes at 25ºC. Absorbance was measured at 517nm. Reaction mixture without test sample served as blank. The free radical scavenging activity was expressed as percentage of inhibition and calculated by the following equation:

\[
\% \text{ inhibition of DPPH activity} = \frac{(A_b - A_s)/A_c)}{X 100\%}
\]

where, \(A_b\) is the absorbance of blank, \(A_s\) is the absorbance of tested samples and \(A_c\) is the absorbance of control samples.

### 2.9.4 NO scavenging activity

Nitric oxide (NO) scavenging assay was performed for the isolated LMW peptides (Jha et al., 2016). Nitric oxide generated from sodium nitroprusside was measured from Greiss reaction (Marcocci et al., 1994). 300µl samples were mixed with 216 µl Greiss reagent (1% sulphanilamide, 2% \(\text{H}_3\text{PO}_4\) and 0.1% NEDH [N-(1-naphthyl ethylenediamine hydrochloride] and 300 µl 5mM sodium nitroprusside prepared in PBS (Phosphate buffer saline) solution. The reaction mixture was incubated for 60 minutes at 25 ºC in a water bath followed by the addition of 2ml water. The absorbance of the samples was taken at 546nm. Radical scavenging activity was calculated as percentage inhibition of NO radical by the following formula:

\[
\% \text{ inhibition of NO radical} = \frac{(A_b - A_s)/A_c)}{X 100\%}
\]

where, \(A_b\) is the absorbance of blank, \(A_s\) is the absorbance of tested samples and \(A_c\) is the absorbance of control samples.

### 2.10 Statistical analysis

Experiments were carried out in completely randomized design (CRD) having three replicates. Data were mean values of three experimental sets and significant values were detected using one way ANOVA (Analysis of Variance) followed by Dunnett’s Multiple Comparison Test which represented values that were significant at \(p<0.05\).

### 3 Results

#### 3.1 Effect of NaCl on growth

NaCl exposure resulted in retarded growth of V. mungo and C. cajan seedlings. Root and shoot lengths were decreased by 22% and 21% in V. mungo and by 46% and 40% in C. cajan seedlings respectively over their control plants. Halopriming of seeds prior to germination narrowed down the rate of inhibition by 18% and 36% in root of V. mungo and C. cajan respectively whereas in shoot the inhibition was reduced to 17% in V. mungo seedlings and by 34% in C. cajan, on an average, over nonprimed control (Figure 1).

![Figure 1: Effect of NaCl on root and shoot growth of 21 days old nonprimed and haloprimed seedlings of Vigna mungo and Cajanus cajan. Values are mean ± SE with three replicates. * indicates statistically significant at \(p\leq0.05\) compared to nonprimed control.](image-url)
3.2 Effect of NaCl on Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{-} contents

Na\textsuperscript{+} and Cl\textsuperscript{-} contents increased while K\textsuperscript{+} contents decreased in root and shoot of both the legume cultivars on NaCl application (Figure 2a, b, c). The accumulation of Na\textsuperscript{+} and Cl\textsuperscript{-} was more in C. cajan than V. mungo. On an average, the uptake of Na\textsuperscript{+} in nonprimed V. mungo and C. cajan increased on an average by 11 and 13-fold respectively in root whereas in shoot, the said increment was by 5-fold in V. mungo and by 8-fold in C. cajan over nonprimed control. Similar trend also was observed for chloride uptake. The chloride uptake, on an average, increased by 168% and 450% in root of V. mungo and C. cajan seedlings respectively whereas in shoot, the uptake increased by 114% and 493% in V. mungo and C. cajan over nonprimed control. Potassium content was found to decrease in root by about 50% in V. mungo and C. cajan by 51% in nonprimed seedlings over control. In shoot of nonprimed seedlings, the said content decreased by 31% in V. mungo whereas the contents were scaled to 21% in C. cajan.

Halopriming of both the cultivars with 50mM NaCl, before their germination in different concentrations of NaCl, altered concentrations of ions in the test cultivars variably. Haloprimed seedlings showed lower uptake of Na\textsuperscript{+} and Cl\textsuperscript{-} ions along with increased accumulation of K\textsuperscript{+} ions. Sodium uptake in haloprimed V. mungo seedlings was found to decrease 7 fold in root and by 4 fold in shoot, on an average, whereas chloride uptake was lowered down on an average to 133% in root and 79% in shoot over nonprimed control. The decrease in potassium contents was lowered to 46% in root and the decrement was further diminished to 22% in shoot of haloprimed V. mungo seedlings, over nonprimed control.

In haloprimed C. cajan seedlings, Na\textsuperscript{+} uptake was lowered down, on an average, by 10-fold in root and by 5-fold in shoot over nonprimed control. Cl\textsuperscript{-} also accumulated in the test seedlings by 363% in root and 121% in shoot on an average over non-primed control. The inhibition in potassium uptake in NaCl primed seedlings of V. mungo diminished to 24% in root and 19% in shoot over nonprimed control attributing better seedling performance.

3.3 HPLC chromatogram

The HPLC chromatogram of the peptide(s) isolated from 21 days old V. mungo and C. cajan seedlings unambiguously discriminated the variation in presence of different content of LMW peptides (0.3 - 5kDa) indicated by varying peak area and peak heights.

From the HPLC chromatograms of the peptides obtained from control seedlings, 150mM salt treated seedlings and haloprimed seedlings, it is evident that in control seedlings, there was maximum expression of peptide at retention time (RT) 5.14 mins in V. mungo which indicates its abundance during seedling growth especially when LMW peptide is concerned. This expression was followed by several other peptides up to RT= 8.5 mins. But, in NaCl treated seedlings the authors speculated that though the peak at RT=5.18 retained, but its abundance was found to decrease by 82% over peptides isolated from control sets. Only two low abundance peaks till before 8.0 minutes were observed in NaCl treated seedlings. This indicated that under salt stress several peptides that usually take part in the growth process of the plant were degraded. In the haloprimed seedlings, the peaks that were found to get degraded in salt treated sets revived at RT’s 7.2 and 8.5 respectively with the highest peak obtained at RT=5.222. The peak at RT=5.222 in haloprimed seedlings, provided an area which was about 4% greater than the area of the same peak obtained from 150mM salt treated seedlings and the inhibition in peptide content was reduced to 80% over control peptides (Figure 3a).
Isolation, purification and partial characterization of low molecular weight peptides of *Vigna mungo* & *Cajanus cajan*

HPLC chromatograms of peptides isolated from control seedlings of *C. cajan* depicted maximal expression and abundance of peptides at RT=5.196. In 150mM NaCl treated seedlings, the abundance of the same peak was found to get reduced by 84% over peptides of control seedlings. This lowering of peptide abundance under NaCl stress probably resulted in the generation of other disintegrated peptide peaks till before RT=8.0. However, peptides isolated from haloprimed seedlings of *C. cajan* exhibited peak area which was 52% more than that of peak area of peptides isolated from 150mM NaCl treated seedlings and also the deterioration in peptide content was reduced to about 78% over control peptides (Figure 3b).

### 3.4 MALDI-TOF analysis

By MALDI-TOF analysis, the spectrum obtained from peptides isolated from 21 days old non-treated or control seedlings of *V. mungo* depicted that several LMW peptides were variably responsible for germination and seedling growth under non-stressed conditions. Noteworthy signals were recorded at m/z values of 3164.095, 3392.275, 3730.246, 3786.693, 3849.599, 3884.410, 3941.740, 4032.702, 4067.151 and 4472.469 representing molecular weight of peptides in Dalton (Da). The peptide having molecular weight of 3786.693Da obtained from control seedlings were prominently expressed (Figure 4a).

For peptides obtained from 150mM NaCl treated seedlings, only one signal was found at m/z value 3810.963Da which represented the molecular weight of the only peptide that could express itself and that too in very low concentration probably due to induced NaCl exposure. The molecular weight of this peptide is very close to the abundant peptide expressed in control seedlings (Figure 4b).

However, spectrum obtained for peptides isolated from haloprimed seedlings of *V. mungo* showed that the abundance of the peptide obtained at m/z 3788.257 (indicative of the molecular weight of the peptide in Dalton) was expressed in abundance.
conferring to better growth of the seedlings under NaCl stress. The molecular weight obtained for the peptide from haloprimed seedlings was also very close to the peptide obtained from control seedlings. Another signal was also obtained at m/z 3731.270 indicating presence of a peptide that was also present in control seedlings having molecular weight of 3730.246Da (Figure 4c).

Similarly, spectrum obtained for peptides isolated from non-treated seedlings of C. cajan exhibited a single m/z signal revealing presence of peptide having molecular weight of 3702.078Da (Figure 5a). The abundance was also satisfactory indicating abundance of peptide during seedling germination and growth. The obtained results could be corroborated with the HPLC generated peaks of same peptide. In NaCl treated seedlings, the abundance of peptide was appreciably reduced as obtained from the m/z signal spectrum indicating molecular weight of the peptide to be 3499.527Da (Figure 5b). Peptides isolated from haloprimed seedlings of C. cajan seedlings again indicated that peptide abundance obtained at m/z signal of 3848.845Da probably responsible for better growth in haloprimed C. cajan seedlings (Figure 5c).

3.5 CD analysis of peptide structures

The LMW peptides isolated from NaCl challenged seedlings acquired lower percentages of secondary structure conformations (alpha helices + beta sheet/strand/turns) over peptides isolated from control seedlings (Table 1). The content of secondary structure decreased to about 51.6% in Vigna and by 24.1% in peptides isolated in Cajanus seedlings. However, peptides isolated from haloprimed test seedlings generated higher percentage of ordered secondary structure, 59.8% in Vigna and 44.5% in Cajanus indicative of stress release under salt stressed conditions. Disordered structure of peptides were found to be higher in salt stressed Cajanus by 57.4% seedlings and less in Vigna by 48.4%. These percentages of disordered conformation decreased to 35.6% in peptides isolated from haloprimed seedlings of Cajanus and to 35.4% in Vigna.

![Image](image_url)

**Table 1 Estimates of Secondary structures in percentages from analysis of Circular Dichroism spectra.**

<table>
<thead>
<tr>
<th>Conformations</th>
<th>Vigna mango</th>
<th>Cajanus cajan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 150mM Nonprimed</td>
<td>Control 150mM Haloprimed</td>
</tr>
<tr>
<td>α-Helix (Regular + distorted)</td>
<td>0.0</td>
<td>4.8</td>
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<tr>
<td>Antiparallel β-sheet (Left twisted+ Relaxed+ Right twisted)</td>
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<td>31.2</td>
</tr>
<tr>
<td>Parallel β-strand</td>
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<td>0.0</td>
</tr>
<tr>
<td>Turn</td>
<td>62.0</td>
<td>28.6</td>
</tr>
<tr>
<td>Others</td>
<td>0.0</td>
<td>35.4</td>
</tr>
</tbody>
</table>
3.6 Bioassay Results

3.6.1 Germination test

Surface sterilized blackgram and pigeon pea seeds were incubated in peptides isolated from their respective seedlings and treatments for 7 days. Of a total of 45 plated seeds, 38 germinated in case of blackgram (Figure 6a) and for pigeon pea it was 32 (Figure 6d). Peptides isolated from nonprimed NaCl stressed seedlings exhibited inhibition in rate of their respective seed germination to about 63% in blackgram (Figure 6b) and by 66% in pigeon pea (Figure 6e) in comparison to the effect of peptides that were isolated from their respective control seedlings. However on exposing seeds of both the cultivars to peptides isolated from their respective haloprimed seedlings depicted better seed germination rate, where the rate of inhibition was appreciably narrowed down to 32% in blackgram (Figure 6c) and 25% in pigeon pea (Figure 6f) respectively over non-primed control.

3.6.2 Stomatal bioassay

Both the legume cultivars revealed that when epidermal peels were exposed to peptides isolated from control seedlings, stomata remained open (Figure 7a, 8a) but in peels exposed to peptides isolated from NaCl stressed seedlings, the guard cells were found to remain flaccid that caused stomatal closure (Figure 7b, 8b). However, when peels were subjected to peptides isolated from haloprimed seedlings, stomatal guard cells gained turgidity that resulted in stomatal opening (Figure 7c, 8c).

Figure 6: Effect of isolated LMW peptides (0.3-5.0 kDa) on seed germination of nonprimed and haloprimed seeds of Vigna mungo (a. control; b. 150mM nonprimed; c. 150mM haloprimed) and Cajanus cajan (d. control; e. 150mM nonprimed; f. 150mM haloprimed).

Figure 7: Effect of LMW peptides (0.3-5.0 kDa) on stomatal regulation in Vigna mungo a: Peptides isolated from control seedlings depicted opening of stomata b. Peptides isolated from salt stressed seedlings revealed complete stomatal closure. c. Application of peptides isolated from haloprimed seedlings exhibited partial opening of stomata.
3.6.3 DPPH radical scavenging activity

DPPH radical scavenging activity was performed to detect the free radical scavenging activities of the peptides isolated from blackgram and pigeon pea (Figure 9). Peptides isolated from control seedlings exhibited radical scavenging activity of about 48% in blackgram and 34% in pigeon pea. In blackgram, the radical scavenging activity of peptide was reduced to about 28% whereas in pigeon pea it decreased to 8%, indicating higher radical scavenging in blackgram than pigeon pea over peptides isolated from their respective control seedlings. However, peptides isolated from haloprimed seedlings exhibited higher radical scavenging activity of about 43% in blackgram and 21% in pigeon pea over its control peptides.

3.6.4 Nitric oxide scavenging activity

It has been demonstrated that under NaCl stress, nitric oxide scavenging activity of peptides obtained from both the cultivars decreased, the inhibition being more in Vigna than Cajanus (Figure 10). In Vigna, peptides isolated from the control seedlings exhibited 25% inhibition in their nitric oxide scavenging activity which was reduced to about 22% in peptides obtained from NaCl stressed seedlings indicating less quenching of radicals. Haloprimed seedlings of Vigna mungo produced peptides that showed higher inhibition of about 26% over the peptides isolated from control plants indicating improved nitric oxide scavenging activity. The peptides obtained from seedlings of Cajanus raised in water exhibited 24% inhibition in nitric oxide scavenging activity. The said inhibition was decreased to 20% in salt stressed Cajanus seedlings indicating lesser scavenging of nitric oxide.
over the activity of its non-primed control peptides. However, peptides from haloprimed seedlings showed 25% inhibition over control peptides indicating greater radical scavenging property of haloprimed peptides.

4 Discussion

After 3 weeks of growth, NaCl induced toxicity resulted in stunted growth of V. mungo and C. cajan seedlings. This indicated salt induced metabolic perturbation was responsible for retarded growth of seedlings. However, when seeds of both the legume cultivars were primed with mild or sublethal dose of NaCl (50mM), increase in root and shoot lengths were recorded, probably an indication of their stress release due to halopriming. Similar report has been published in salt stressed V. radiata (Saha et al., 2010), N. sativa (Gholami et al., 2015) and Lolium perene (Tilaki & Behtari, 2017).

Under NaCl stress, Na\(^+\) and Cl\(^-\) contents increased in the seedlings of salt stressed V. mungo and C. cajan. Accumulation of Na\(^+\) and Cl\(^-\) ions were more in C. cajan than V. mungo. Uptake of Na\(^+\) ions facilitated Cl\(^-\) ions to enter the roots down the chemical gradient (Moussa, 2004). Similar results were reported in salt-stressed V. radiata and Broussonetia papyrifera (Saha et al., 2012; Zhang et al., 2013). Under optimum physiological conditions, K\(^+\) ions are known to contribute to 6 -10% of the plant dry weight (Raven et al., 1976; Shin, 2014) and serves as a major inorganic osmolyte (Shabala & Cuin, 2008). It plays essential role in regulating several physio-biochemical processes of which stomatal opening and closing is crucial for maintenance of undisturbed synthesis of photoassimilates within plants (Shabala, 2003; Hasanuzzaman et al., 2018). NaCl exposure in the test seedlings resulted in reduced K\(^+\) contents and increased Na\(^+\) contents thereby causing stomatal regulation to get hampered significantly in both the legume cultivars as Na\(^+\) ions failed to replace the beneficial aspect of K\(^+\) ions. This happened because sodium ions competed with Na\(^+\)-K\(^+\) co-transporter under salinity (Zhu, 2003; Almeida et al., 2017).

Halopriming of seeds of V. mungo and C. cajan with 50mM NaCl resulted in decreased uptake of Na\(^+\) and Cl\(^-\) ions and increased accumulation of K\(^+\) ions in both the cultivars under 150mM NaCl exposure compared to non-primed control. Present outcomes are in agreement with the results obtained by seed preconditioning in salt stressed mungbean (Saha et al., 2012). This occurred due to preferential uptake of K\(^+\) ions over Na\(^+\) ions into the xylem element (Carden et al., 2003). Moreover, halopriming of seeds stimulated Na\(^+\)/H\(^+\) antiporter exchange activity of SOS1 protein pump as in Hordeum vulgare which promoted exclusion of Na\(^+\) from the root cells of haloprimed seedlings.

Small peptides play beneficial roles in dictating intercellular and intracellular signalling cascades related to growth and metabolism in plants (Matsubayashi & Sakagami, 2006). Therefore, current research was aimed at isolation of ultra-filtered LMW peptides from both the legume cultivars which were subjected to HPLC and MALDI-TOF analysis. This aided not only in determining the peptide occurrences but also to compare their variations in content during their normal seedling germination and under salt stressed conditions. Further role of halopriming in alleviating the salt induced adversities could also be deciphered. HPLC chromatograms of peptides isolated from both the test seedlings portrayed that in non-primed control seedlings, maximum peptide expression and occurrence took place during normal seedling growth. However, peptides obtained from NaCl stressed seedlings generated chromatogram that showed lesser peptide abundance compared to non-primed control seedlings. This probably indicated that several LMW peptides which usually participate in growth processes of plants were degraded under salinity. Halopriming of seeds helped to overcome such adversities to considerable extent in salt stressed conditions as indicated by increased peak area and reappearance of a few peaks that was noted in control seedlings. This reveal that ameliorative efficacy of halopriming lead to better growth and development of both the pulses under NaCl stressed environments. Matrix-assisted laser desorption ionization (MALDI) being a soft source of molecule(s) ionisation was utilized to generate fragmentation of isolated peptide(s) thereby helping to determine their molecular weight (Castañeda-Ovando et al., 2012). For both the tested materials, molecular weight of the most prominent peptide obtained from the m/z signals of MALDI spectrum was found to be close to each other. Intensities of this prominent peak showed a trend that was similar to its occurrence in HPLC spectra for both cultivars. The appearance of signal having m/z 3731.270 in MALDI spectra of peptides from haloprimed blackgram seedlings was similar to that of peptide signal at m/z 3720.246 derived from its control seedlings. This indicated that halopriming resulted in reappearance of that LMW peptide facilitating better germination and growth of haloprimed seeds than directly salt treated seeds. Although no such peptide reappearances were noted in MALDI spectrum of C. cajan but, role of halopriming was evident from the increased peak intensity of haloprimed peptides over direct salt treated ones. Thus, seed halopriming in both the cultivars imparted better growth of primed seeds under salt stressed conditions due to prior salt acclimation.

Circular dichroism spectroscopy helped to determine percentages of secondary structure adopted by the peptides of both the pulses under salt stress and how these conformations altered in case of haloprimed peptides under salt exposure. Secondary conformations were found to decrease under NaCl induced desiccation manifesting deformation and distortion in peptide structure usually involved in normal plant growth and developmental processes. This reflected salt induced denaturation.
of peptides, formation of more flexible, unfolded, unordered structures which affected the equilibrium between the contents of α-helical and β-sheet structures. Moreover, unordered structures increased in peptides obtained from salt stressed seedlings probably due to protein aggregation as it was observed in whey protein isolates under temperature and pH variations (Topmcyznska-Mleko et al., 2014). But, in haloprimed peptides the percentages of secondary conformations of peptides were much more regularized over peptides of salt stressed seedlings. Higher percentages of secondary conformations and lower percentages of disorder probably helped the primed seeds to impart efficient metabolism under salinity stress in both the pulses.

The above obtained results were validated by bioassays performed with the isolated LMW peptides from both the cultivars. Bioassays helped to demonstrate the putative effects of respective peptides on seed germination and stomatal regulation and thereby seedling growth from which the authors could get some notion regarding their role on growth regulation in the test seedlings. Abundance of LMW peptide from control seedlings of both the varieties, promoted seed germination rate and kept stomatal pore open for CO₂ assimilation. This could be correlated with the higher K⁺ ion accumulation in shoots and satisfactory antioxidative activities of LMW peptides favouring luxuriant growth of both cultivars. Similar antioxidative properties have been reported in LMW isolated from mulberry plants (Jha et al., 2014). But, the peptides isolated from NaCl challenged seedlings of both varieties narrowed down the germination rate, failed to arouse turgidity in guard cells due to decreased uptake of K⁺ ions in shoot thereby resulting in stomatal shut down and hindered CO₂ fixation. Decrease in free radical scavenging activities of these peptides derived from NaCl stressed seedlings is also in agreement with the above physiochemical events causing stunted seedling growth in both the varieties. Similar effect has been reported in Schizonepeta tenuifolia when exposed to high salt doses at and above 75mM NaCl (Zhou et al., 2018). But, peptides obtained from haloprimed seedlings exhibited improved seed germination, increased turgidity of stomatal guard cells causing stomatal opening and carbon assimilation under NaCl stress. This could be corroborated with increased accumulation of K⁺ ions in shoots of haloprimed cultivars and antioxidative activity of LMW peptides that resulted in improved growth in both the varieties on salt exposure. However, the scavenging activity of peptides was found to be more efficient in V. mungo than C. cajan. This indicated more stress tolerance capability of blackgram over pigeon pea under NaCl stress, when seeds were haloprimed.

Therefore, adoption of cost effective halopriming technique by poor farmers may help to boost legume production by improving crop stand in saline prone agricultural lands to partially meet up with the food crisis of the flourishing world population.

Conclusion

To conclude authors relate that under NaCl stress, growth was inhibited in both blackgram and pigeonpea seedlings due to lower abundance of LMW peptides. The isolated peptides exhibited antioxidative properties that effectively scavenged reactive oxygen species when present in plenty. The peptides were stress-responsive and increased on seed halopriming when compared to that of salt stressed sets. Peptides isolated from haloprimed V. mungo had better antioxidative properties than those isolated from haloprimed C. cajan which could be correlated with improved growth in V. mungo as recorded by the authors. Secondary structural conformations were highly hampered under salt stress. But haloprimed V. mungo and C. cajan seeds showed improved physiological performances indicating that adopting this cost effective halopriming technique helped to overcome salt induced adversities under salt exposure. Moreover, this technique may help to increase legume production in saline prone agricultural lands to meet up with the food demands of increasing population.

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Conflicts of Interest and financial disclosure

The authors declare that no conflict of interest exists. This work was supported by a Research Grant No. 1012(Sanc.)/ST/P/S&T/2G-2/2013 obtained from the Department of Science and Technology, Government of West Bengal.

References


SCREENING OF SHORT DURATION RICE GENOTYPES FOR ZINC EFFICIENCY

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ABSTRACT

Zn (Zn) deficiency in tropical soils affects nutritional quality of food grains and to secure nutritional quality, one potential option is the ‘agronomic bio-fortification’ which depends mainly on genotypic efficiency to absorb and accumulate more of Zn into grains. To identify the rice genotypes having better ability to absorb and translocate more Zn into grains, a field experiment was conducted with 15 short duration rice genotypes on a Zn deficient sandy clay loam soil with and without Zn addition. A split plot design was adopted using Zn treatment in main plots, as M1: control (only recommended dose of N, P, and K without Zn) and M2: recommended dose of N, P, and K with Zn (100 kg ZnSO4 ha⁻¹ as soil application + 0.5% as foliar application thrice at 50% flowering, milk and dough stages) and the selected genotypes as sub plots. Results of present study revealed that, Zn application significantly increased the average grain yield (13.5%) and grain Zn content (37 to 55 %) over control. The rice genotypes, CO 47 performed better with higher grain yield of 5980 and 6750 kg ha⁻¹ respectively under both with and without Zn fertilization. Higher grain yield index was noted with CO 47, and CO 51 (92.7) followed by ADT 36 (88.0) while the highest grain Zn uptake index was noted with CO 47 (40.4). Based on the yield and Zn uptake efficiency, the genotypes CO 47, CO 51, ADT 36, ADT 37, MDU 5, MDU 6, TKM 12, IR 50 were found efficient and responsive to Zn fertilization thus can be utilized for Zn bio-fortification. The rice genotypes TPS 5, Anna 4, CB 14508 are highly inefficient and susceptible to Zn deficiency which needs Zn fertilization without it the yield loss in unavoidable.

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1 Introduction

Micronutrients such as Zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), boron (B), and molybdenum (Mo) are essential but required in small quantities for the growth and development of many plants (Alloway, 2008). These micronutrients are very crucial for plant, animal and human health and play a key role in various biochemical processes. It is estimated that Zn deficiency in Indian soils is likely to increase from 49 to 63% by 2025. A substantial genetic variability in tolerance to Zn deficiency exists in many crops and this variability has been used significantly to improve the Zn acquisition by plants (Sudha & Stalin, 2015a). The inherent soil micronutrients status mainly depends on the geological substrate and weathering processes (White & Zasoski, 1999). Though the total concentration of micronutrients generally vary in soils due to diversity in parent materials, their availability to plants depends on pH, organic matter, texture, soil moisture and clay mineralogy (Martens & Lindsay 1990; Fageria, 2001). Generally, micronutrient content is high in the surfaces soils and decreases with depth (Gupta, 2005). The soil pH and oxidation-reduction conditions decide micronutrient form in which these are present in soil and determine their bioavailability by influencing fixation reactions (Fageria et al., 2011). Moreover, their content in the soils is also influenced by the type of cultivated crops which differ in their uptake of micronutrients. In the tropical soils of India, micronutrient deficiencies in soils is attributed to the larger removal from soil by fertilizer responsive high yielding crops, use of micronutrient free high analysis fertilizers, mono cropping, increased cropping intensity and reduction in the use of organic manures etc.

Zinc is considered as the fourth most important yield-limiting nutrient after, nitrogen, phosphorus and potassium (Shukla et al., 2018) and plays an important role in starch formation, synthesis of auxin and essential for the production of growth hormones like IAA. It also influences RNA levels and ribosome contents in cells, component of protein and chloroplast and also necessary for various enzymes that are responsible for driving many metabolic reactions in plants. In addition, Zn is a co-factor for many enzymes and proteins involved in cell division, nucleic acid metabolism and protein biosynthesis (Figueredo, et al., 2012).

Continuous intensive cropping along with high yielding crop varieties has further aggravated the depletion of soil Zn leading to low Zn concentration in edible grains. The reported use efficiency of micronutrients in Indian soils seldom exceeds 5 per cent (Shukla et al., 2014). Reliance on cereal-based diets with low Zn may induce Zn deficiency in human beings resulted in serious health problems such as growth retardation, susceptibility to infectious diseases, hypogonadism, iron deficiency anemia, and poor birth outcome in pregnant women (Graham et al., 2000; Graham et al., 2012). The potential options to correct micronutrient deficiency in human beings may be food supplementation, food fortification or bio-fortification (White & Broadley, 2009). “Agronomic bio-fortification” cannot be successfully achieved through the application of fertilizers nutrients which is proved effective in enriching micronutrients content in rice grain by controlling the availability of soil micronutrients (White & Broadley, 2009). Studies showed that certain plant species, as well as their genotypes exhibit a significant genetic variation in their tolerance to Zn deficiency and this ability of a genotype to grow and yield well in a Zn deficient soil is termed as “Zn efficiency” (Hacisalihoglu & Kochian, 2003). Genotypic differences for Zn use efficiency have been reported in several crops species (Rengel, 2005; Cakmak et al., 2010) and related to various mechanisms operating in the rhizosphere and within the plant system.

Rice is an important staple food and energy source for more than half of the world population, however, it is a poor source of essential micronutrients such as Fe and Zn (Welch, 2008; Tripathy et al., 2017). It is highly sensitive crop to Zn deficiency with drastic yield reduction and lesser Zn concentration in the grains yield when Zn is limited in soils. Application of Zn fertilizers is essential for keeping sufficient available Zn in soil solution and maintaining adequate transport during critical growth stages of crop which leads increased grain Zn concentration to a considerable amount. Thus the present study was proposed to study the effect of Zn fertilization on grain yield and Zn content in rice and to identify the Zn uptake efficient short duration rice genotypes for bio-fortification.

2 Materials and Methods

2.1 Experimental site

Field experiment was conducted in naturally Zn deficient soil at wetland farms of Tamil Nadu Agricultural University, Coimbatore. The farm is situated at an altitude of 426.72 m above MSL in the Western agro climatic Zone of Tamil Nadu at 11° North latitude and 77° East longitude.

2.2 Climatic condition

Annual rainfall of 641 mm in 37 rainy days and cropping period rainfall of 302 mm in 18 rainy days were recorded during 2016-17 at the principal observatory, Agro Climate Research Centre, Tamil Nadu Agricultural University, Coimbatore. The North East Monsoon (NEM) contributes 375 mm of rainfall. The relative humidity ranged from 26 to 94 per cent with mean sunshine hours of 7.4 hours. The mean maximum and minimum temperatures prevailed during the cropping period was 30.3°C and 21.3 °C which were favourable for growth and development of rice crop (AMFU - Coimbatore, 2017).
2.3 Soil characteristics

Surface soil samples were drawn from 0-15 cm air dried, crushed and passed through two mm sieve. The initial soil properties were analyzed as per the standard methods which were furnished in table 1. The soil of the experimental field was slightly alkaline in pH (8.32) with low salt (0.30 dS m⁻¹) and available N status (222 kg ha⁻¹), medium organic carbon (5.60 g ha⁻¹), high in available P (24.0 kg ha⁻¹) and K (410 kg ha⁻¹). The soil of the study area was deficient to Zn (0.98 mg ha⁻¹) but sufficient for other micronutrients.

2.4 Experiment details

The experiment was conducted during the kharif season of 2017 at wetland farms of Tamil Nadu Agricultural University, Coimbatore to screen short duration rice genotypes (15 nos.) for higher grain Zn content and Zn efficiency using soil and foliar Zn fertilization practices. Rice genotypes included in the study were ADT 36, ADT 37, ADT 43, ADT (R) 45, MDU 5, MDU 6, CO 47, CO 51, TPS 5, ASD 16, TKM 12, Anna (R) 4, IR 50, CB 14508 and Shabhagithan. Two Zn treatments viz., M₁: NPK Control (150: 50: 50 kg NPK ha⁻¹) and M₂: M₁+ Zn at 100 kg ZnSO₄ ha⁻¹ as soil application + 0.5% as thrie foliar application at 50% flowering, milk and dough stages. The fertilizers were applied basally and the crop was grown to maturity and harvested. The grain and straw yield of crop, Zn content and uptake was recorded beside the crop was grown to maturity and harvested. The grain and straw yield of crop, Zn content and uptake was recorded beside the crop was grown to maturity and harvested. The efficiency of 7200 kg and 6980 kg ha⁻¹ respectively. Application of 150:50:50 kg +100 kg ZnSO₄ ha⁻¹ as soil application + 0.5% as foliar application thrice at 50% flowering, milk and dough stages recorded the highest grain and straw yield of 7200 kg and 9297 kg ha⁻¹. This increase in yield might be attributed to the greater influence of Zn on basic plant life processes, such as nitrogen metabolism (uptake of nitrogen and protein quality) and photosynthesis (chlorophyll synthesis and carbonic anhydrase activity) (Hazra et al., 2015; Kumar et al., 2018). The genotype CO 47 registered the highest grain yield in both control (6000 kg ha⁻¹) and Zn applied plots (7200 kg ha⁻¹), while the lowest grain yield was recorded in IR 50 in control (4500 kg ha⁻¹) and Zn applied treatments (5142 kg ha⁻¹). However with regard to straw yield, highest straw yield was recorded with

Table 1 Details of analytical procedures employed in soil analysis

<table>
<thead>
<tr>
<th>Estimations</th>
<th>Procedure</th>
<th>Reference</th>
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<td>Jackson (1973)</td>
</tr>
<tr>
<td>Electrical conductivity (EC)</td>
<td>1:2.5 soil water suspension</td>
<td>Jackson (1973)</td>
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<tr>
<td><strong>Chemical properties</strong></td>
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<tr>
<td>Organic carbon</td>
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<td>Walkley &amp; Black (1934)</td>
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<td>Olsen et al. (1954)</td>
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CO 47 and the lowest yield was evident in MDU 5. The overall data revealed 13.5% increase in grain yield and 11 per cent increase in straw yield due to Zn application over NPK alone and the yield increase in genotypes might be due to increased activity of enzymes and auxin metabolism in the plant which was reported by Sudha & Stalin (2015a).

### 3.2 Effect of Zn fertilization on Zn content

Crop species have differential micronutrient density in grains when grown under similar conditions and the Zn concentration ranged from 11.5 to 37.2 mg kg\(^{-1}\) and 22.8 to 41.4 mg kg\(^{-1}\) in whole rice grains and straw respectively in Zn applied treatment and the increase in Zn content was 37 to 55 per cent over control. In the present study also genotypes differed significantly in Zn absorption and CO 47 recorded the maximum Zn concentration in both grain and straw (37.2 and 41.4 mg kg\(^{-1}\)) while the lowest Zn content was recorded in CB 14508 (11.5 mg kg\(^{-1}\)) and TPS 5 (22.8 mg kg\(^{-1}\)) in grain and straw respectively (Table 3). It was statistically at par with CO 51(35.6 and 40.7 mg kg\(^{-1}\)) and ASD 16 (35.4 and 40.1 mg kg\(^{-1}\)) in grain and straw Zn contents respectively. The difference in micronutrient concentration in rice genotypes may be attributed to micronutrient loading in grains and according to Impa et al. (2013), some genotypes showed continued root uptake which is the predominant source of Zn loading in grain, whereas in some genotypes, net remobilization of Zn from shoot and root to grain was predominant. Further, the difference was also might be due to genotype and environment interaction, water management and soil factors like pH, EC, organic carbon, phosphorus and micronutrient availability (Sudha & Stalin, 2015a). Increased root proliferation further increased the Zn uptake, enhanced synthesis of carbohydrates and its transport to grain (Sudha & Stalin, 2015b; Tripathy et al., 2017).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Yield (kg ha(^{-1}))</th>
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<tr>
<td>MDU 5</td>
<td>4500</td>
</tr>
<tr>
<td>CO 47</td>
<td>6000</td>
</tr>
<tr>
<td>CO 51</td>
<td>5850</td>
</tr>
<tr>
<td>TPS 5</td>
<td>5000</td>
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<tr>
<td>CB 14508</td>
<td>5317</td>
</tr>
<tr>
<td>ASD 16</td>
<td>5167</td>
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<td>ADT 43</td>
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<tr>
<td>Shabhagithan</td>
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<td>IR 50</td>
<td>4492</td>
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<th>SE(m)</th>
<th>SE(d)</th>
<th>CD (P=0.05)</th>
<th>SE(m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
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<td>113.2</td>
<td>39.9</td>
<td>55.5</td>
<td>111.4</td>
</tr>
<tr>
<td>V</td>
<td>154.5</td>
<td>310.1</td>
<td>109.2</td>
<td>152.2</td>
<td>305.2</td>
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<td>M X V</td>
<td>218.5</td>
<td>438.6</td>
<td>154.5</td>
<td>215.1</td>
<td>431.7</td>
</tr>
</tbody>
</table>

M = Zn application | V = Rice genotypes
3.3 Effect of Zn fertilization on Zn uptake

Zn application had positive influence on the Zn uptake by rice grains varied from 58 to 96.6 g ha\(^{-1}\) in control and 153 to 269 g ha\(^{-1}\) in Zn applied plot in grain yield while in straw it ranged from 179 to 242 in control and from 274 to 385 g ha\(^{-1}\) in Zn applied treatment (Table 4). Genotypes exerted their differential response on Zn removal, in which CO 47 recorded higher Zn uptake (269 and 385 g ha\(^{-1}\)) and the lowest Zn uptake was witnessed in IR50 in both grain and straw (153 and 274 g ha\(^{-1}\)). Total Zn uptake by rice genotypes ranged from 241 to 324 g ha\(^{-1}\) in control treatment and 428 to 654 g ha\(^{-1}\) in Zn applied treatment. The increased in Zn uptake could be attributed to better Zn absorption and root to shoot transport by Zn efficient genotypes and also probably due to more efficient transport system such as ion channel or ion pump, compared with the Zn-inefficient genotypes (Malewar et al., 1993; Kumar et al., 2018).

### 3.4 Zn Efficiency

To assess the Zn efficiency of rice genotypes, yield and Zn uptake index was worked out by taking the ratio between yield and uptake in control and Zn applied treatments (Figure 1). The grain yield index varied from 76 to 92.7, grain Zn index ranges from 34.6 to 46.5 and grain Zn uptake index varied from 26.3 to 40.4. The results showed that, CO 47 and CO 51 had higher grain yield index (92.7) followed by ADT 45 (88.0) while the highest grain Zn uptake index was noted with CO 47 (40.4). The lowest grain yield index of 76.0 and grain Zn uptake index of 26.3 were recorded in TPS 5, whereas the lowest grain Zn index of 34.2 was noticed in ADT 45. This was perhaps due to the abundant supply of Zn nutrition and balanced NPK, which increased the protoplasmic constituents, accelerates the process of cell division and elongation, photosynthesis processes, respiration, nitrogen metabolism-protein synthesis, other biochemical and physiological activates (Sudha & Stalin, 2015a).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Grain Zn content (mg kg(^{-1}))</th>
<th>Straw Zn content (mg kg(^{-1}))</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>(+Zn)</td>
</tr>
<tr>
<td>Anna (R) 4</td>
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<tr>
<td>ADT 37</td>
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<td>34.3</td>
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<tr>
<td>MDU 5</td>
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<td>33.7</td>
</tr>
<tr>
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<td>16.2</td>
<td>37.2</td>
</tr>
<tr>
<td>CO 51</td>
<td>14.9</td>
<td>35.6</td>
</tr>
<tr>
<td>TPS 5</td>
<td>11.6</td>
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<tr>
<td>CB 14508</td>
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<td>TPS 5</td>
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<tr>
<td>Shabbagithan</td>
<td>12.7</td>
<td>31.1</td>
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<tr>
<td>IR 50</td>
<td>14.2</td>
<td>31.7</td>
</tr>
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<td>ADT (R) 45</td>
<td>11.8</td>
<td>34.5</td>
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<tr>
<td>MDU 6</td>
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<td>32.5</td>
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<th>SE(m)</th>
<th>CD (P=0.05)</th>
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<td>0.82</td>
</tr>
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<td>V</td>
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<td>1.98</td>
<td>0.69</td>
<td>1.13</td>
<td>2.26</td>
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<td>M X V</td>
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<td>2.80</td>
<td>0.98</td>
<td>1.59</td>
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</table>

M = Zn application  V = Rice genotypes
### Table 4 Effect of Zn fertilization on the Zn uptake by rice genotypes

<table>
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<tr>
<th>Genotypes</th>
<th>Grain (-Zn)</th>
<th>Grain (+Zn)</th>
<th>Zn uptake (g kg⁻¹)</th>
<th>Straw (-Zn)</th>
<th>Straw (+Zn)</th>
<th>Total (-Zn)</th>
<th>Total (+Zn)</th>
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<td>204</td>
<td>319</td>
<td>282</td>
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<td>343</td>
<td>302</td>
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<td>MDU 5</td>
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<td>208</td>
<td>301</td>
<td>276</td>
<td>472</td>
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</tr>
<tr>
<td>CO 47</td>
<td>96.6</td>
<td>269</td>
<td>227</td>
<td>385</td>
<td>324</td>
<td>655</td>
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<tr>
<td>CO 51</td>
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<td>239</td>
<td>204</td>
<td>374</td>
<td>292</td>
<td>614</td>
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<td>316</td>
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<td>536</td>
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<td>583</td>
<td></td>
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</tr>
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<td>349</td>
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<td>534</td>
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<td>Shabbagithan</td>
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<td>179</td>
<td>315</td>
<td>252</td>
<td>490</td>
<td></td>
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<tr>
<td>IR 50</td>
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<td>274</td>
<td>333</td>
<td>429</td>
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<td>ADT (R) 45</td>
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<td>239</td>
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<tr>
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<td>289</td>
<td>287</td>
<td>460</td>
<td></td>
</tr>
<tr>
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<td>195</td>
<td>210</td>
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<td>282</td>
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<th>SE(d)</th>
<th>CD (P=0.05)</th>
<th>SE(m)</th>
<th>SE(d)</th>
<th>CD (P=0.05)</th>
<th>SE(m)</th>
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<td>4.41</td>
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<td>32.2</td>
<td>11.3</td>
<td>19.2</td>
<td>38.6</td>
<td>13.6</td>
</tr>
</tbody>
</table>

M = Zn application  \ V = Rice genotypes

Figure 1 Grain yield and Zn uptake indices in rice grain
The Zn uptake and efficiency varied widely among the genotypes which might be due to the ability of rice genotypes to increased Zn availability in the rhizosphere for subsequent uptake by releasing active Zn mobilizing substance such as phytosiderophores. Similar results with the resistant rice varieties under Zn stress condition in extracting Zn from complex and organically bound Zn forms was reported by Jin et al. (2008), However the susceptible varieties absorbed Zn only from readily available sources.

Based on the yield and Zn uptake efficiency, grouping of genotypes was made into efficient responsive, efficient non responsive, inefficient responsive and inefficient non responsive (Table 5 & Figure 2) as per the classification of Fageria & Baligar (1993) for nutrient use efficiency and average yield at low Zn supply. The first group comprised of efficient and responsive genotypes that produced more than the average yield of all the genotypes under Zn deficiency condition and their Zn efficiency was also higher than the average efficiency. Among the genotypes it was found that genotypes ADT 36, ADT 37, MDU 5, MDU 6, Co 47, CO 5, TKM 12 and IR 50 were grouped under efficient responsive.

The second group of efficient and non-responsive genotypes produced more than average yield at low Zn supply but response to Zn application was lower than the average. The genotypes ADT 45 and Shabagithan are fund to be efficient non responsive. Third category, inefficient and responsive genotypes which produced less than average yield, but their response to Zn application was above the average. The genotypes falls this category are ADT 43 and ASD 16 and the fourth group of genotypes produced less than average yield and also less than average response to added Zn. These genotypes are inefficient and non-responsive. The genotypes, TPS 5, Anna 4 and CB 14508 were grouped under inefficient non responsive genotypes. This indicates that genotypes with high efficiency are desired as they will be efficient scavengers of Zn under low level of Zn supply. Similar findings were reported by Kumar et al., (2018) in rice.

### Table 5 Genotypes categorization based on yield and uptake efficiency

<table>
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<tr>
<th>Genotypes</th>
<th>Responsive</th>
<th>Non responsive</th>
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<tbody>
<tr>
<td>Efficient</td>
<td>ADT 36, ADT 37, MDU 5, MDU 6, Co 47, CO 5, TKM 12, IR 50</td>
<td>ADT 45, Shabagithan</td>
</tr>
<tr>
<td>Inefficient</td>
<td>ADT 43, ASD 16</td>
<td>TPS 5, Anna 4, CB 14508</td>
</tr>
</tbody>
</table>
Conclusions

The present investigation is concluded that, basal soil application of 150: 50: 50 kg NPK + 100 kg ZnSO₄ ha⁻¹ along with 0.5% as foliar spray thrice during 50% flowering, milk and dough stages of the rice crop significantly increased the rice grain and straw yield, Zn content and its uptake. Based on the efficiency indices, the genotypes, CO 47, CO 51, ADT 36, ADT 37, MDU 5, MDU 6, TKM 12, IR 50 were found efficient and responsive to Zn fertilization thus can be utilized for bio-fortification of Zn. The genotypes ADT 45 and Shabbiagatan are found to be Zn efficient but non-responsive to Zn application thus indicates that they are genetically efficient to utilize the native soil Zn thus suitable for Zn low condition. The rice genotypes TPS 5, Anna 4, CB 14508 are highly inefficient and susceptible to Zn deficiency.

Acknowledgement

The authors gratefully acknowledge Tamil Nadu Agricultural University for implementing the project by providing sufficient facilities.

Conflict of interest

The corresponding author declares that there is no conflict of interest.

References


AMFU - Coimbatore (2017) Annual report of Agrometerological field unit, Agro climate research centre, Tamil Nadu Agricultural University, Coimbatore.


MOLECULAR AND FLORAL CHARACTERIZATION OF MAINTAINER AND RESTORERS IN NEWLY DEVELOPED RICE (Oryza sativa L.) HYBRIDS

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ABSTRACT

Hybrid rice is one of the successfully demonstrated technologies, which is feasible and readily adoptable for enhancing the rice production. But availability of stable cytoplasmic male sterility and fertility restoring system is vital for commercial exploitation of heterosis in rice. In the present study, seven testers were identified based on their pollen and spikelet fertility at Hybrid rice section, Mandya which were validated for fertility restoration (Rf) locus by four (RM 1, RM 6100, RM 6344 and RM 1108) reported linked SSR markers before hybridization with 10 CMS lines to develop 70 hybrids. All the four markers showed highly positive association with phenotypic fertility restoration and maintainer type allele. The markers RM 6100, RM 6344 and RM 1108 linked to Rf4 locus located on chromosome 10 and 7. Based on pollen fertility, 23 hybrids were found to be very effective restorers and 47 as partial restorers while, 44 hybrids were found to be very effective restorers and 26 hybrids showed partial restorer behavior based on spikelet fertility. The testers, MSN 36, KMR 3, PBK 093-1-4-4-2-1, PBK 095-5-4-5-1 and PBK 091-3-7-1-1 were classified as effective restorers for all the CMS lines.

KEYWORDS
Cytoplasmic male sterility
Maintainer
Pollen fertility
Restorer
Spikelet fertility
SSR markers

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1 Introduction

Rice (*Oryza sativa* L.) is an important staple food crop for more than half of the world population including India. Over 90 per cent of the rice is produced and consumed in Asia accounting for more than 65 per cent of calorific intake (Khush, 2004). The Green revolution enabled the rice production to meet the growing demand of the increasing population and to maintain self-sufficiency thus helped many countries to escape from starvation (Sharma et al., 2013). The increase in production has to be achieved from 106.3 million tonnes to 120 million tonnes by the year 2020 without adversely affecting the environment has there is a drastic decline and deterioration of resources such as land, water and other inputs (Sharma et al., 2013).

In order to increase production with the pace of growing population, Chinese scientists successfully demonstrated the feasibility of exploiting hybrid vigour in rice by adopting three line system based on the cytoplasmic male sterility fertility restorer system (Hwa & Yang, 2008). It has potential to bridge yield gap and to meet the challenge of increasing rice production while sustaining the natural resource base. Hybrid rice technology exploits the phenomenon of hybrid vigour (heterosis) to increase the yield potential of rice varieties by 15% to 20% over current commercial cultivars (Virmani & Edwards, 1983; Hwa & Yang, 2008). The commercial success of hybrid rice in China has clearly demonstrated the potential of this technology by yielding on an average of 1 to 1.5 tonnes more grain per hectare than the conventional high yielding varieties (Matthayatthaworn et al., 2011).

For developing high yielding heterotic hybrids, the first step is to identify restorers that can efficiently restore the fertility of *F₁*. The process of screening for the trait of fertility restoration is laborious and time consuming as it involves test crossing with a set of CMS lines and evaluation of *F₁* for pollen and spikelet fertility. Identification of maintainer and restorer lines by observation of pollen fertility and spikelet fertility in test crosses involving CMS lines is the commonly practised traditional approach. In general, identification of different restorer lines for different CMS sources helps in increment of diversity.

Recently, molecular markers have also been successfully used by several researchers for the tagging of fertility restorer genes and classification of pollen parents into the categories of sterility maintainers and fertility restorers (Sheeba et al., 2009). The use of molecular markers linked to *Rf* genes can enhance the selection efficiency, save time and avoid the complications associated with phenotype-based screening. The fertility restorer genes *Rf*-3 and *Rf*-4 for WA-type CMS have been mapped on chromosome 1 and 10, respectively (Sheeba et al., 2009). The genes controlling fertility restoration do not behave identically under different genetic backgrounds because of which different segregation ratios are obtained in different combinations of CMS and restorer lines.

In order to evolve hybrids that are superior in yield potential to the existing ones and adaptable in newer areas, one essential component of the requisite methodology is to identify newer potential restorer and maintainer lines from locally adopted material through systematic generation of new crosses and their proper evaluation. Therefore, there is a need to identify an effective restorer line having tightly linked *Rf* genes, so that marker aided selection can be used as a tool to identify restorers at an early stage of crop growth, there by such identified lines can be utilized to effect hybrids in the very present season. Keeping these points in view, present study was planned to characterize newly developed lines into maintainer and restorers based on floral fertility and SSR markers markers linked to fertility restorer gene.

2 Material and Methods

The experimental material comprised of 70 newly developed hybrids by crossing ten CMS lines (Table 1) along with seven testers (Table 2) in line × tester mating design at Hybrid rice

Table 1 List of CMS lines along with parentage and source of cytoplasm

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>CMS lines</th>
<th>Parentage</th>
<th>Cytoplasmic source in CMS line (A line)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMS 1A</td>
<td>KCMS 40A</td>
<td>CMS – WA</td>
</tr>
<tr>
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<td>KCMS 48A</td>
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<td>IR 68888A/Pragathi</td>
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</tr>
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<td>CMS 4A</td>
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<tr>
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<td>CMS 7A</td>
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</table>

Table 2 List of testers used along with parentage

<table>
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<td>7</td>
<td>PBK 091-3-7-1-1-1</td>
<td>MSN- 98/ Athira</td>
</tr>
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</table>
section, ZARS, V. C. Farm, Mandya, Karnataka, India during summer 2014. These seven testers were selected based on the pollen and spikelet fertility studies from 100 newly developed parental lines from earlier studies.

Leaf sample from all the male parents (Table 2) were used for DNA isolation and validation of SSR markers linked to fertility restoration (Rf) locus. Total genomic DNA was extracted from 35 days old seedling using CTAB (Cetyl Trimethyl Ammonium Bromide method) (Saghai-Marooof, 1984). Quality and quantity of extracted DNA was determined spectrophotometrically by taking absorbance at 260 nm and 280 nm (Varian Cary 50 Spectrophotometer). The extracted DNA samples were diluted using TE buffer to get the working concentration of 50ng/µl. The diluted DNA samples were used for SSR analysis.

Earlier reported tightly linked markers were used for genotyping of testers. List of SSR markers used in the study with their features is presented in Table 3. For SSR analysis PCR conditions were optimized as described previously (Panaud et al., 1996) with minor modifications. PCR amplification reaction were conducted in 20-25 ng of template DNA, 100µM of dNTPs, 100 pmolhs of each of forward and reverse primer, 10x PCR buffer (10mMTris pH 8.0, 50 mMKci, 1.8 mM MgCl2 and 0.01 mg/ml gelatin) and one unit of Taq DNA polymerase (Bangalore Genei, India) in a volume of 20 µl. One drop of mineral oil (Sigma) was dropped on each well of reaction mixture to avoid evaporation in the thermocycler. The PCR amplification was carried out using a thermocycler (Eppendorf) with an initial denaturation step of 94°C for 4 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing depending on the primers used (30 SEC at 56°C) and extension (2 min at 72°C). After the last cycle, a final extension was carried out for 7 min at 72°C. The reproducibility of amplification products was checked twice for each primer. Amplified products were resolved by electrophoresis in 2.5% agarose gel containing 0.5 µg/ml ethidium bromides. Four µl of loading dye was added to 20 µl of PCR products and mixed well before loading into the wells. A 10 µl of the PCR products were loaded into the wells, care was taken to prevent mixing of samples between the wells. A voltage of 1 -5 v/cm was given for a time period of three hours for separation of PCR fragments. After electrophoresis, the DNA banding pattern was viewed under UV light and documented. The bands generated by microsatellite primers were given score M for maintainer alleles and R for restorer type alleles. CRMS 32B and MSN 36 were used as standard check for identification of maintainer and restorer allele, respectively.

These 70 hybrids developed were evaluated along with their parents and three standard checks viz., KRH-2, KRH-4 and GK 5013 which are high yielding medium duration hybrids. Seedlings were transplanted with a spacing of 20 cm x 15 cm in single rows with single seedling per hill in a Randomized Complete Block Design (RCBD) with two replications during Kharif 2014. All the recommended package of practices was followed timely to ensure good crop establishment. Observations were recorded on five randomly selected competitive plants on pollen fertility (%) and spikelet fertility (%).

2.1 Estimation of pollen fertility

For pollen fertility (%), three spikelets, one each from top, middle and bottom of main panicle of CMS lines each from two replications were collected and pollen grains were squeezed out from all the anthers on a clean glass slide and stained with 1.0 per cent I-KI (Iodine-Potassium Iodide) stain (which is prepared by dissolving 2 g of potassium iodide and 1g of iodine in 100 ml of distilled water) and examined under microscope at a magnification of 10X. The pollens were considered to be fertile if they were round, plumpy and deeply stained, while they were considered as sterile if they were shrunk, unstained and irregular in shape. Three microscopic fields were counted for each spikelet and pollen fertility was expressed in percentage.

\[ \text{Pollen fertility} = \frac{\text{Number of fertility (stained) pollen grains}}{\text{Total number of pollen grains}} \times 100 \]

Table 3 List of SSR markers used for validation of fertility restoration (Rf) locus in rice

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Marker</th>
<th>Allele</th>
<th>Chromosome No</th>
<th>Genetic distance</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM1</td>
<td>Rf3</td>
<td>1</td>
<td>5.6 cM</td>
<td>F: GCGAAAAACACAAATGCAAAAAA R: GGTGTGTTGGGACCTTGAC</td>
<td>Alavi et al. (2009)</td>
</tr>
<tr>
<td>2</td>
<td>RM 6100</td>
<td>Rf4</td>
<td>10</td>
<td>1.2 cM</td>
<td>F: TCTCTTCCAGTACCCCGAC R: GCTGGATCACAGATCATTTGCA</td>
<td>Ahmadikah et al. (2007)</td>
</tr>
<tr>
<td>3</td>
<td>RM 6344</td>
<td>Rf4</td>
<td>7</td>
<td>13.3 cM</td>
<td>F: ACCGCATGGGATATGAC R: TGCCATCAGTACCTTTCACC</td>
<td>Sheeba et al. (2009)</td>
</tr>
<tr>
<td>4</td>
<td>RM 1108</td>
<td>Rf4</td>
<td>10</td>
<td>1.6 cM</td>
<td>F: GGCAGAATCGATCCGAC R: CTGGATCTGGGACAGGAC</td>
<td>Bazrkar et al. (2008)</td>
</tr>
</tbody>
</table>
The genotypes were grouped into four different classes based on pollen fertility per cent (Virmani et al., 1997).

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Class</th>
<th>Range</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maintainer (M)</td>
<td>0 to 1.00 per cent</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Partial maintainer (PM)</td>
<td>1.1 to 20.00 per cent</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Partial restorer (PR)</td>
<td>20.1 to 80.00 per cent</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Effective restorer (R)</td>
<td>80.1 to 100.00 per cent</td>
<td></td>
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</tbody>
</table>

### 2.2 Estimation of spikelet fertility

Spikelet fertility percent is the ratio of number of filled grains to total number of spikelets per panicle and expressed as percentage. Number of filled and chaffy spikelets in five main panicles of five selected plants in each replication were pooled together and averaged at the time of harvest to assess the spikelet fertility.

\[
\text{Spikelet fertility} \, \% = \frac{\text{Number of filled grains}}{\text{Total number of spikelets}} \times 100
\]

The genotypes were grouped into four different classes based on spikelet fertility per cent as shown under (Virmani et al., 1997).

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Class</th>
<th>Range</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effective maintainer (M)</td>
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</tr>
<tr>
<td>2</td>
<td>Partial maintainer (PM)</td>
<td>5.1 to 20.00 per cent</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Partial restorer (PR)</td>
<td>20.1 to 70.00 per cent</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Effective restorer (R)</td>
<td>70.1 to 100.00 per cent</td>
<td></td>
</tr>
</tbody>
</table>

### 3 Results and discussion

Identification of maintainers and restorers is fundamental for the commercial exploitation of heterosis breeding programme using cytoplasmic male sterility (CMS) system (Sharma et al., 2012; Bhati, 2017). Restorers for different cytosterile sources will increase the cytoplasmic diversification, which in turn can prevent genetic vulnerability due to the use of single CMS source. Therefore, the present investigation was undertaken to identify maintainers and restorers based on molecular and floral characterization.

Staining patterns and shapes of pollen grains in genotypes possessing male sterility-inducing cytoplasm and sterility-maintaining nuclear gene(s) are known to be influenced by the developmental stage at which pollen abortion occurs and these developmental stages are related to nuclear stages (Chaudhary et al., 1981; Eikonin & Tsvetova 2012). Based on staining pattern obtained, the pollen parents are classified as effective restorers (MSN 15-16) and partial restorers (PBK 095-5-4-5-1) (Figure 1). The highest pollen and spikelet fertility was observed in MSN -15-16 (92.68) and MSN – 36 (92.75) respectively (Table 4).

Four reported SSR markers linked (Table 3) to fertility locus (R) were used to screen the seven male parents with CRMS 32B and MSN 36 as standard for maintainer and restorer allele respectively. All the four markers (RM 1, RM 6100, RM 6344 and RM1108) exhibited polymorphism between standard restorer

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**Figure 1** Pollen fertility restoration patterns in testers. The harvested panicles were fixed in aceton: alcohol (1:3) solution. Anthers were smeared in solution containing 0.5% iodine in 2% potassium iodide and examined under light microscope.

**Table 4** Phenotypic and genotypic scoring of testers based on pollen fertility, spikelet fertility and for four SSR markers

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Genotypes</th>
<th>Pollen fertility</th>
<th>Phenotypic scoring</th>
<th>Spikelet fertility</th>
<th>Genotypic scoring</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Per cent</td>
<td>Class</td>
<td>Per cent</td>
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<tr>
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<td>MSN – 36</td>
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<td>92.75</td>
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<tr>
<td>2</td>
<td>KMR -3</td>
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<td>R</td>
<td>79.84</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>MSN -71</td>
<td>87.80</td>
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<td>84.51</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>MSN -15-16</td>
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<td>R</td>
<td>90.12</td>
<td>R</td>
</tr>
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<td>PBK 093-1-4-4-2-1</td>
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<td>88.33</td>
<td>R</td>
</tr>
<tr>
<td>6</td>
<td>PBK 095-5-4-5-1</td>
<td>74.12</td>
<td>PR</td>
<td>70.61</td>
<td>R</td>
</tr>
<tr>
<td>7</td>
<td>PBK 091-3-7-1-1</td>
<td>86.78</td>
<td>R</td>
<td>81.34</td>
<td>R</td>
</tr>
</tbody>
</table>

Note: R- Restorer PR- Partial restorer
(MSN 36) and maintainer (CRMS 32B) (Table 4, Figure 2). The allelic pattern of all the four polymorphic markers was compared with phenotypic reaction of fertility based on pollen fertility and spikelet fertility across CMS lines. All the four markers (RM 1, RM 6100, RM 6344 and RM 1108) showed highly positive association with phenotypic fertility restoration and maintainer type allele (Table 5). The markers RM 6100, RM 6344 and RM 1108 are linked to Rf4 locus which is located on chromosome 10 and 7 reported by Sheeba et al. (2009) and Bazrkar et al. (2008), respectively and RM 1 linked to Rf3 locus which is located on M-CRMS 32B (maintainer), 1: MSN-36, 2: KMR-3, 3: MSN-71, 4: MSN-15-16, 5: PBK 093-1-4-4-2-1, 6: PBK 095-5-4-5-1, 7: PBK 091-3-7-1-1

Figure 2 Screening of polymorphic markers across the male parent

Table 5 Association of marker allele with the phenotype based on pollen/spikelet fertility across ten CMS lines

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Genotypes</th>
<th>CMS 1A</th>
<th>CMS 2A</th>
<th>CMS 3A</th>
<th>CMS 4A</th>
<th>CMS 5A</th>
<th>CMS 6A</th>
<th>CMS 7A</th>
<th>CMS 8A</th>
<th>CMS 9A</th>
<th>CMS 10A</th>
<th>Genotypic scoring</th>
</tr>
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<tbody>
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<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>KMR – 3</td>
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<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
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<td>MSN - 71</td>
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<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>M</td>
</tr>
<tr>
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<td>MSN -15-16</td>
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<td>R</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
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<td>PR</td>
<td>M</td>
</tr>
<tr>
<td>5</td>
<td>PB 1-4-4-2-1</td>
<td>PR/R</td>
<td>PR/R</td>
<td>PR</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
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<td>PR/R</td>
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<td>PR/R</td>
<td>R</td>
<td>PR/R</td>
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<td>PR/R</td>
<td>R</td>
<td>M</td>
</tr>
<tr>
<td>7</td>
<td>PB 3-7-1-1</td>
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<td>PR</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
<td>R</td>
<td>PR</td>
<td>R</td>
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</table>

M- Maintainer R- Restorer PR- Partial restorer PM- Partial maintainer

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chromosome 1 reported by Alavi et al. (2009) and Ahmadikhah et al. (2007). These results indicated that Rf3 and Rf4 locus are the predominant restoration alleles for CMS lines studied.

Seventy hybrids produced by crossing promising ten CMS lines with seven male parents which were selected from parental lines based on spikelet fertility and pollen selection and reported markers linked to Rf locus were evaluated during Kharif 2014. The results obtained based on pollen and spikelet fertility is presented in Table 6. The genotypes were classified as maintainer, restorer, partial maintainer and partial restorer based on pollen and spikelet fertility. Based on pollen fertility, 23 hybrids were found to be very effective restorers and 47 as partial restorers. The testers, MSN 71 and MSN 15-16 behaved as partial restorers for all the CMS lines except CMS 2A. Based on spikelet fertility, 44 hybrids were found very effective restorers and 26 hybrids showed partial restorer behavior (Table 6).

Table 6 Classification of crosses into restorers and maintainers based on pollen fertility and spikelet fertility

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Hybrids</th>
<th>Pollen fertility</th>
<th>Spikelet fertility</th>
<th>Based on both</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Percent Class</td>
<td>Percent Class</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CMS 1A × MSN 36</td>
<td>76.22 PR</td>
<td>71.71 R</td>
<td>PR/R</td>
</tr>
<tr>
<td>2</td>
<td>CMS 1A × KMR -3</td>
<td>94.06 R</td>
<td>90.05 R</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>CMS 1A × MSN -71</td>
<td>35.97 PR</td>
<td>27.10 PR</td>
<td>PR</td>
</tr>
<tr>
<td>4</td>
<td>CMS 1A × MSN -15-16</td>
<td>62.15 PR</td>
<td>57.10 PR</td>
<td>PR</td>
</tr>
<tr>
<td>5</td>
<td>CMS 1A × PB 1-4-4-2-1</td>
<td>77.85 PR</td>
<td>75.05 PR</td>
<td>PR/R</td>
</tr>
<tr>
<td>6</td>
<td>CMS 1A × PB 5-4-5-1</td>
<td>79.26 PR</td>
<td>74.78 R</td>
<td>PR/R</td>
</tr>
<tr>
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<td>CMS 1A × PB 3-7-1-1</td>
<td>69.02 PR</td>
<td>61.92 PR</td>
<td>PR</td>
</tr>
<tr>
<td>8</td>
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<td>74.02 R</td>
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<tr>
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<td>PR</td>
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<tr>
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<td>22.79 PR</td>
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<tr>
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<td>63.05 PR</td>
<td>60.05 PR</td>
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<td>19</td>
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<td>77.06 PR</td>
<td>73.83 R</td>
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<td>76.93 PR</td>
<td>73.08 R</td>
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M- Maintainer, R- Restorer, PR- Partial restorer, PM- Partial maintainer

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Table 6: Contd…..

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Hybrids</th>
<th>Pollen fertility Percent</th>
<th>Class</th>
<th>Spikelet fertility Percent</th>
<th>Class</th>
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<td>R</td>
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<td>R</td>
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M- Maintainer, R- Restorer, PR- Partial restorer, PM- Partial maintainer
Based on pollen fertility, the lines CMS 5A and CMS 7A had highest number (4) of effective restorers, followed CMS 9A which had three effective restorers. While, the lines CMS 1A, CMS 3A and CMS 8A had least number of restorers (one) and CMS 6A had all partial restorers. Based on spikelet fertility, CMS 2A and CMS 5A had highest number (6) of restorers followed by CMS 4A and CMS 9A had five effective restorers each. CMS 1A, CMS 6A, CMS 7A and CMS 10A as they had four effective restorers each. The male parents, MSN 36, KMR 3, PBK 093-1 - 4-4-2-1, PBK 095-5-4-5-1and PBK 091 -3-7-1 -1 were classified as effective restorers for all the CMS lines and MSN 71 and MSN 15-16 showed partial restoration (Table 5).

However, majority of male parents restored complete fertility in CMS 2A followed by CMS 5A and CMS 7A. The effective restorers, identified in this study could be used to develop hybrids for commercial purpose. The results also proved the effectiveness of marker aided selection of restorers as most of testers selected produced perfect restoration in hybrids. However, problem of partial restoration need to be solved. Similar opinion was given by Rosamma & Vijaykumar (2005).

**Conclusion**

The results clearly indicated that, fertility restoration reaction of the genotypes varies with genetic background of CMS lines. The identified maintainers and restorers are locally adopted. The identified restorer lines can be used as pollen parent in developing new commercial hybrid varieties in rice breeding programme. It is also proven from the results that markers are more effective and precise in selection of restorers as most of testers selected produced perfect restoration in hybrids.

**Conflicts of interest**

The authors declare that there is no conflict of interest for the contents of the manuscript.

**Reference**


LEVERAGING TRADITIONAL CROPS FOR FOOD AND FEED: A CASE OF HULLESS BARLEY (HORDEUM VULGARE) LANDRACES IN ETHIOPIA

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ABSTRACT

This study explored food-feed traits in genotypes of 25 indigenous Ethiopian landraces, 13 landraces introduced into Ethiopia and 5 local checks of hulless barley (Hordeum vulgare). The genotypes were evaluated for straw fodder quality traits and the traits were related to grain yield and straw yield. The genotypes were grown in Ethiopia during the 2016 cropping season using augmented design consisting of 5 complete blocks. Results of the study showed high genotypic variability in grain yield (5.1 t/ha), straw yield (7.03 t/ha) and straw content of crude protein (CP: 29.1 g/kg), neutral detergent fiber (NDF: 77 g/kg), acid detergent fiber (ADF: 41 g/kg), acid detergent lignin (ADL: 22.7 g/kg) and in vitro organic matter digestibility (IVOMD: 72 g/kg). Further, cluster analysis determined 6 genotypes i.e. 243231, 241790, 219177, 243235, 241787, 241789 among Ethiopian landraces that showed food-feed traits with an average of 3.44 t/ha of grain, 5.64 t/ha of straw and 55.9 g/kg of CP. The correlation between grain yield with straw yield and nutritive value parameters was insignificant. Principle component analysis determined that either CP, NDF or IVOMD can express the nutritive value of hulless barley straw. The study highlights the natural genotypic variation in grain yield and straw traits in hulless barley that can be exploited using appropriate breeding methods to develop varieties with a combination of food traits for human food and feed traits for livestock feed. These varieties would be particularly beneficial for mixed crop-livestock systems that are predominant in developing countries.

KEYWORDS
Food-feed
Genotypic variation
Landraces
Hulless barley
Nutritive value
Straw

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1 Introduction

Barley (Hordeum vulgare L.) is one of the earliest domesticated crops (Mamo et al., 2014) and the fourth most important cereal in terms worldwide production (FAO, 2016). More than half of land area under barley crop is in developing countries (Grando & Macpherson, 2005). In Ethiopia, barley is among the oldest cultivated crops and has been grown for at least 5,000 years, in a wide range of agro-ecologies (Mamo et al., 2014). It has high economic and social importance as human food, malt for brewing and animal feed (Kaso & Guben, 2015). Most barley varieties are hulled, however, hulless barley (hulless barley) is gaining preference due to the ease with which it can be processed, prepared and presented for food (Zohary & Hopf, 2000). Barley occupies about 959,000 hectares of land with total production of 2,025,000 tones (CSA, 2016). In the predominantly mixed crop-livestock systems of Ethiopia, the potential contribution of barley straw to the feed supply of livestock is significant. A grain yield of 3 t/ha of barley is associated with approximately 4 t of straw (Cooper et al., 2001) which could feed a 300 kg cow for 800 days (calculation based on Kearl, 1982). However, barley straw, with an inherently low nutritive value (38 g/kg CP, 6 MJ/kg ME and 27 g/kg 0.75 dry matter intake) (Heuzé et al., 2016), cannot cover maintenance requirements of the cow over that period (Goodchild, 1997). The trend that straws represent an increasingly important part of total crop value has been reported (Kelley et al., 1991). However, new improved varieties and cultivation methods have been reported to lead to decrease in straw yields (Austin et al., 1980; Riggs et al., 1981). Rejection of improved varieties because of poor straw traits has been reported in barley (Capper et al., 1986; Capper et al., 1988). In India, wheat farmers requested wheat breeders to consider straw yield in wheat improvement programs (Schiere et al., 2004). Traxler & Byerlee (1993) reported that the economic value of straw is an important criterion in the adoption of new cereal varieties by small holder mixed crop-livestock farmers. Accordingly, the development of high grain yielding varieties of food and malt barley by the International Center for Agricultural Research in Dry Areas (ICARDA), which holds the world mandate for barley, needs to consider straw traits. ICARDA has reported on the possibility of breeding for dual purpose barley with high forage yield as well as high grain yield for the Mediterranean region where green stage barley grazing is practiced. Studies to simultaneously boost grain yield and straw nutritive value traits of cereal and grain legume crops are ongoing at ICARDA. Several studies have reported on the possibility of improving grain yield alongside straw traits of lentil (Alkhtib et al., 2017), chickpea (Wamatu et al., 2017), maize (Eritiro et al., 2013) and pearl millet (Blümmel et al., 2010). A focus on dual purpose hulless barley for high grain yield, high straw yield and high nutritive value would be particularly relevant for regions in Asia and Sub-Saharan Africa where straw feeding to livestock is commonly practiced. Landraces are still the backbone of agricultural systems in many developing countries as they are characterized by high genetic heterogeneity and good adaptation to local environmental conditions (Cecarelli & Grando, 1996). We hypothesize that there is a possibility to find hulless barley landraces which combine superior food and feed traits. The Ethiopian gene bank collection on hulless barley germplasm consists of landraces indigenous to Ethiopia, henceforth referred to as ETH landraces, and those introduced from other regions, henceforth referred to as introduced landraces. This study aims to characterize for grain yield and straw traits and to identify the food-feed relations in Ethiopian landraces of hulless barley for use in future breeding work on dual-purpose barley.

2 Materials & Methods

2.1 Experimental material

A total of 43 hulless barley germplasms which included 25 ETH landraces obtained from the Ethiopian Biodiversity Institute (initially collected from 11 administrative zones), 13 introduced landraces originally obtained from the gene bank of ICARDA and 5 local checks obtained from Holetta Agricultural Research Center (HARC), Ethiopia were obtained for the study (Table 1).

2.2 Experimental site

Trials were conducted at HARC (9° 3’ N, 38° 30’ E, altitude 2400 m.a.s.l), during the main cropping season of 2016 (July - December) under rainfed conditions. Mean maximum and minimum temperatures during the study were 22.1 and 6.2°C respectively. The experiment was laid out in an augmented randomized complete block design (Federer & Ragavarao, 1975) consisting of 5 blocks in which ETH landraces and introduced landraces were planted in un-replicated plots and 5 local checks genotypes were replicated 5 times to estimate experimental error variance. Plot size was 2.5 m length and 0.4 m between rows. Fertilizer was applied at a rate of 50/100 kg/ha, urea/DAP. Trial were managed as per recommended practice for barley cultivation. At physiological maturity, plots were manually harvested from 2 areas (1.6 m2) laid over 2 middle rows of each plot. After sun-drying and threshing of biomass, representative samples from each plot were analysed for chemical composition and digestibility.

2.3 Straw quality analysis

Oven-dried (100°C; 24 h) samples were ground, sieved through a 1mm mesh and analysed using a combination of conventional laboratory analysis and Near Infrared Spectroscopy (NIRS; Foss
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</table>

ETH: Ethiopian, ICARDA: International Center for Agricultural Research in Dry Areas, NA: not available
Forage Analyser 5000 with software Package WinISI II in 1108-2492 nm spectra range). A basal NIRs calibration was developed and validated by wet chemistry analyses of 20% representative samples. For conventional analyses, dry matter (DM) and crude protein (CP) were determined as per procedures of AOAC (2005). Crude protein was calculated from nitrogen by multiplication with the factor of 6.25. Cell wall fractions namely neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were determined as described by Van Soest et al. (1991). In vitro organic matter digestibility was measured in rumen microbial inoculum using in vitro gas production technique (Menke & Steingass, 1988) and calculated using the equation below suitable for roughages as described by Menke et al. (1979).

IVOMD (g/kg) = 14.88+0.889*GP+0.45*CP+0.0651*XA

Where GP: 24 h net gas production (ml/200 mg); CP: Crude protein (g/kg DM); XA: Ash content (g/kg DM).

2.4 Statistical analysis

Data was analysed using SAS version 12.1 Software (SAS, 2012). A mixed model was used for analysis of variance as follows

\[ Y_{ij} = M + A_i + B_j + E_{ij} \]

Where: \( Y_{ij} \) is response variable; \( M \) is general mean, \( A_i \) is the fixed effect of the \( i \)th standard checks and random effect of genotypes, \( B_j \) is the random effect of \( j \)th block and \( E_{ij} \) is the random error. The relationship between grain yield and straw traits was determined using Pearson correlation.

To quantify the contribution of major determinants (CP, NDF, IVOMD) of nutritive value of straw, principal component analysis (PCA) was carried out using standardized data. The signs and magnitudes of the eigenvectors were examined for relevance. Relevance was based on the facts that NDF is negatively correlated to DM intake (Horrocks & Vallentine, 1999) and IVOMD is positively correlated to metabolizable energy (ME). Results from the PCA determined which nutritive parameters would be included in cluster analysis. Cluster analysis was used to classify the genotypes into homogeneous groups/clusters depending on similarity in grain yield, straw yield and straw nutritive value parameters. Values of pseudo F statistics and Hotellin’s pseudo T2 statistics were used to identify the optimum number of clusters. Cluster analyses was carried out using standardized data. Standardized data was used in principle component analysis and cluster analysis to unify units of measurement.

3 Results

3.1 Grain yield and straw yield

Table 2 and 3 present results of descriptive analyses and analysis of variance of grain and straw traits for hulless barley landraces. There were significant (\( P<0.05 \)) variations in grain yield among local checks, ETH landraces, but not among introduced landraces. Combined means of grain yield of ETH landraces were significantly higher than local checks and introduced landraces. Grain yield ranges were 0.473 - 5.49 t/ha among ETH landraces, 0.184 - 1.68 t/ha among introduced landraces and 1.16 - 5.63 t/ha among local checks. Considering all genotypes in this study, the magnitude of range in grain yield was 4.92 t/ha. The minimum yielding genotype was found in ETH landraces while the
maximum yielding genotype was found within local checks. Variation in straw yield in ETH landraces and local checks genotypes was significant (P<0.05). ETH landraces and local checks were not significantly different. Combined mean of straw yield of ETH landraces was higher (P<0.05) than introduced landraces. Range in straw yield was 0.863 - 7.9 t/ha, 0.64 - 4.98 t/ha and 1.5 - 9.32 t/ha in ETH landraces, introduced landraces and local checks respectively. The minimum and maximum straw yielders were found in introduced landraces and local checks respectively. Considering all genotypes, the difference in yield was 8.46 t/ha.

3.2 Straw nutritive value

Table 2 and 3 present results of descriptive analysis and analysis of variance of straw traits. Variation in CP and cell wall constituents was significant (P<0.05) among the 3 groups. In vitro organic matter digestibility varied significantly among local checks and introduced landraces but not for ETH landraces. This indicated that CP and NDF of ETH landraces were significantly higher (P<0.05) than introduced landraces. The difference between ETH landraces and checks in NDF was insignificant. Means of ADF, ADL and IVOMD of ETH landraces were significantly higher than introduced landraces but less than local checks. The range of CP was 30 - 59 g/kg, 25.7 - 50.2 g/kg and 24.3 - 45.6 g/kg in ETH landraces, introduced landraces and local checks respectively. The range of NDF was 781 - 858 g/kg, 717 - 836 g/kg and 706 - 860 g/kg within landraces, introduced landraces and local checks respectively. The range of ADF was 530 - 617 g/kg, 484 - 588 g/kg and 501 - 624 g/kg in ETH landraces, introduced landraces and local checks respectively. The range of ADL was 74.6 - 97.3 g/kg, 56.4 - 89.8 g/kg and 72.5 - 107 g/kg in ETH landraces, introduced landraces and local checks respectively. The range of IVOMD was 400 - 472 g/kg, 385 - 487 g/kg and 362 - 460 g/kg in ETH landraces, introduced landraces and local checks respectively. Genotypes which had the lowest and the highest CP were found in local checks and ETH landraces respectively. The lowest and highest genotypes in terms of NDF were found in local checks. The lowest and highest genotypes regarding ADF and ADL were found in introduced landraces and local checks respectively. Genotypes with the lowest and highest IVOMD were found in local checks and introduced genotypes respectively. Considering all genotypes, the magnitude of range in CP, NDF, ADF, ADL and IVOMD was 34.7 g/kg, 154 g/kg, 176 g/kg 50.6 g/kg and 125 g/kg respectively.

3.3 Principal component analysis

Principle component analysis generated 3 principle components (Table 4). Principle component 1 explained 71.1%. majority of the variability of nutritive value of straw. PC1 best expressed the nutritive value of straw because an examination of eigenvectors showed that CP and IVOMD had positive signs suggesting they would contribute positively to nutritive value of straw while NDF had negative sign suggesting it would contribute negatively to the nutritive value of straw. The magnitude of eigenvectors was almost similar, 0.558, -0.566 and 0.606 for CP, NDF and IVOMD respectively, which implies that either of the eigenvectors can be used to represent the nutritive value of barley straw. Therefore, CP was included in cluster analysis because it represents the nutritive value of straw and it is a critical parameter considering that straws of cereals are known to have low CP contents.

3.4 Cluster analysis based on food-feed traits

Cluster analysis grouped the 43 genotypes into 5 clusters based on grain yield and straw traits (Table 5). The number of genotypes distributed across each cluster was as follows: 9, 17, 6, 5 and 6 in cluster 1, 2, 3, 4 and 5 respectively. Cluster 1 was dominated by

### Table 3 Descriptive statistics for grain yield and straw traits of hulless barley genotypes

<table>
<thead>
<tr>
<th>Variable</th>
<th>ETH landraces</th>
<th>Introduced landraces</th>
<th>Local checks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>Grain yield (t/ha)</td>
<td>3.11</td>
<td>0.473</td>
<td>5.49</td>
</tr>
<tr>
<td>Straw yield (t/ha)</td>
<td>4.29</td>
<td>0.863</td>
<td>7.9</td>
</tr>
<tr>
<td>CP</td>
<td>45.3</td>
<td>30</td>
<td>59</td>
</tr>
<tr>
<td>NDF</td>
<td>815</td>
<td>781</td>
<td>858</td>
</tr>
<tr>
<td>ADF</td>
<td>576</td>
<td>530</td>
<td>617</td>
</tr>
<tr>
<td>ADL</td>
<td>84.6</td>
<td>74.6</td>
<td>97.3</td>
</tr>
<tr>
<td>IVOMD</td>
<td>440</td>
<td>400</td>
<td>472</td>
</tr>
</tbody>
</table>

CP: crude protein (g/kg DM), NDF: neutral detergent fiber (g/kg DM), ADF: acid detergent fiber (g/kg DM), ADL:acid detergent lignin (g/kg DM), IVOMD: in vitro organic matter digestibility (g/kg), ETH: Ethiopian.
LeveRaging Traditional Crops for Food and Feed: A Case of Hulless Barley Landraces In Ethiopia

3. Correlation between food yield and straw traits

ETH landraces, local checks and introduced landraces had different food-feed correlation profiles (Table 6). No correlation between grain yield and straw yield or grain yield and nutritive traits was found in both ET and introduced landraces. Grain yield in introduced genotypes, correlated moderately and positively to

3.5 Correlation between grain yield and straw traits

Table 6 Correlation coefficients between grain yield and straw traits

<table>
<thead>
<tr>
<th>Straw traits</th>
<th>ETH landraces</th>
<th>Introduced landraces</th>
<th>Local checks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straw yield</td>
<td>0.611</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CP</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NDF</td>
<td>ns</td>
<td>ns</td>
<td>0.429</td>
</tr>
<tr>
<td>ADF</td>
<td>ns</td>
<td>ns</td>
<td>0.426</td>
</tr>
<tr>
<td>ADL</td>
<td>ns</td>
<td>ns</td>
<td>0.568</td>
</tr>
<tr>
<td>IVOMD</td>
<td>ns</td>
<td>ns</td>
<td>-0.641</td>
</tr>
</tbody>
</table>

CP: crude protein (g/kg DM); NDF: neutral detergent fiber (g/kg DM); ADF: acid detergent fiber (g/kg DM); ADL: acid detergent lignin (g/kg DM); IVOMD: in vitro organic matter digestibility (g/kg); ns: P>0.05 otherwise P≤0.05.

introduced landraces (88%). ETH landraces dominated cluster 2 representing 65% of the total genotypes. Cluster 3 was equally dominated by the three groups of genotypes. Cluster 4 mainly constituted of introduced landraces and local checks (80%). All genotypes in cluster 5 were ETH landraces. Cluster 5 had the highest grain yield, straw yield, CP, IVOMD compared to other clusters.

Table 5 Cluster means of major food and feed traits of hulless barley

<table>
<thead>
<tr>
<th>Cluster</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total genotypes (N)</td>
<td>9</td>
<td>17</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>N of landraces</td>
<td>5</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>N of introduced</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>N of local check</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Food-feed traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grain yield (t/ha)</td>
<td>2.58(80.3)</td>
<td>2.26(56.3)</td>
<td>1.44(85.2)</td>
<td>2.83(58.7)</td>
<td>3.44(44.5)</td>
</tr>
<tr>
<td>Straw yield (t/ha)</td>
<td>2.53(57.2)</td>
<td>3.88(38.1)</td>
<td>3.38(55.7)</td>
<td>3.36(22.9)</td>
<td>5.64(29.4)</td>
</tr>
<tr>
<td>CP (g/kg)</td>
<td>37.9(2.8)</td>
<td>46.6(5.04)</td>
<td>33.3(4.41)</td>
<td>28.4(7.38)</td>
<td>55.9(4.13)</td>
</tr>
<tr>
<td>NDF (g/kg)</td>
<td>804(3.2)</td>
<td>790(5.25)</td>
<td>827(0.6)</td>
<td>836(2.13)</td>
<td>792(1.14)</td>
</tr>
<tr>
<td>IVOMD (g/kg)</td>
<td>438(2.18)</td>
<td>446(4.67)</td>
<td>418(5.11)</td>
<td>411(5.24)</td>
<td>447(2.75)</td>
</tr>
</tbody>
</table>

CP: Crude protein, Value between parentheses denotes coefficient of variation, N: number of.

Table 4 Principle component analysis of the nutritive parameters of hulless barley straw

<table>
<thead>
<tr>
<th>Statistics</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eigenvalue</td>
<td>2.13</td>
<td>0.525</td>
<td>0.341</td>
</tr>
<tr>
<td>Variation explained (%)</td>
<td>71.1</td>
<td>17.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Eigenvectors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>0.558</td>
<td>0.738</td>
<td>-0.379</td>
</tr>
<tr>
<td>NDF</td>
<td>-0.566</td>
<td>0.672</td>
<td>0.475</td>
</tr>
<tr>
<td>IVOMD</td>
<td>0.606</td>
<td>-0.05</td>
<td>0.793</td>
</tr>
</tbody>
</table>

straw yield while it did not correlate to nutritive value parameters of straw. There was no correlation between grain yield and straw yield and grain yield and CP in local checks. Grain yield correlated positively and moderated to cell wall constituents and negatively and strongly to IVOMD.

4 Discussion and conclusions

Wide genetic range in grain yield, straw yield and nutritive value was found among ETH landraces, introduced landraces and local checks. Furthermore, combined data from all genotypes showed wider ranges indicating the possibility to improve both grain yield and straw traits by simple selection. Generic variation in grain yield and straw traits was also observed in maize (Ertiro et al., 2013), in chickpea (Wamatu et al., 2017) and in lentil (Alkhtib et al., 2017; Wamatu et al., 2017). Crude protein content in feeds is important to achieve optimum rumen activity in addition to ensuring adequate dry matter intake of feed. A level of 70-80 g/kg CP and 100-110 g/kg CP are recommended for non-lactating and lactating cows respectively. The highest level of CP among the genotypes in the study was 59 g/kg. However, CP content of crop residues can be improved through agronomic practices, particularly by applying a feasible level of nitrogen fertilization (Blümmel et al. 2007; Mosisa et al. 2007). Dry matter intake of low-quality roughages is closely and negatively associated with NDF content (Horrocks & Vallentine 1999). Wide genotypic variation in NDF content of barley straw was found in this study, indicating that dry matter intake of barley straw could be improved by exploiting natural variability in straw content of NDF. However, dry matter intake is affected by other factors such as physical and morphological properties of feed and species of livestock. Thus, it is important to test palatability of straws of newly developed hulless barley genotypes before release. Interaction between genotype and location in straw traits has been reported in maize (Ertiro et al., 2013). Thus, more studies are needed to determine genotype-environment interactions in hulless barley.

Principle component analysis showed that CP, NDF and IVOMD coefficients had similar magnitude, suggesting that nutritive value of hulless barley straw can be presented using either CP, NDF or IVOMD. Increasing the nutritive value of barley straw by breeding requires efficient screening of large numbers of genotypes for straw quality. Neutral detergent fibers and CP are simpler to be determined compared to IVOMD. Thus, one of them could be used to express the nutritive value of the straw. Breeders can improve straw quality by targeting to increase CP and IVOMD or decrease NDF. Similar results were reported by Alkhtib et al. (2017) in lentil and Wamatu et al. (2017) in field pea. However, a simpler method is still required. It has been reported that botanical structure of faba bean straw can be used to screen genotypes for straw nutritive value (Alkhtib et al., 2016). Thus, studies on predicting the nutritive value of barley straw depending on botanical structure may be useful. The correlation between grain yield and straw traits was insignificant in both ETH landraces and introduced landraces. Grain yield correlated moderately to straw yield but not to straw nutritive value parameters, indicating that improving nutritive value of ETH landraces and introduced landraces would not be associated with a decline in grain yield. Grain yield correlated positively to cell wall constituents and negatively to IVOMD. That implies that improving nutritive value of the straw should be done consciously. Weak correlations between food and feed traits were also reported in Ertiro et al. (2013) in maize, Blümmel et al. (2007) in pearl millet and Blümmel et al. (2010) in sorghum. Cluster analysis indicated that 6 ETH landraces found in cluster 5 had superior grain yield and straw traits compared to other clusters, suggesting that selecting ETH landraces for food-feed traits holds promise.

Wide genetic variation in grain yield and straw traits in hulless barley implies high possibility to develop genotypes of hulless barley which combine superior grain yield and straw traits. ETH landraces could be a potential genetic pool for any effort to improve both grain yield and straw traits. However, variability in straw nutritive value should be confirmed for use by livestock. That could include botanical structure and physical traits of straw. The effect of the environment on performance of hulless barely genotypes in terms of food and feed traits should be determined. More studies are also needed to identify inheritance of straw traits. That will assist crop breeders to design appropriate approaches to develop dual purpose genotypes of hulless barley.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References


UTILIZATION OF WHEAT STRAW FOR THE PRODUCTION OF ASPARAGINASE IN SOLID-STATE FERMENTATION

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KEYWORDS
Asparaginase
Fusarium oxysporum
Optimization
Wheat straw
Solid-state fermentation

ABSTRACT
Asparaginase is one of the most widely used industrial enzymes of therapeutic significance; in present study an attempt had been undertaken to synthesize asparaginase from an inexpensive and abundantly available wheat straw agro-waste by solid-state fermentation of Fusarium oxysporum NCIM 1008. The essential fermentation variables were optimized to enhance the microbial growth and enzyme activity. The maximal asparaginase yield (21.54 U/gds) was reported with 60% (v/w) initial moisture content, pH 6.0, supplemented with 0.75% L-asparagine and incubated at 30 °C for 96 h. Present investigation clearly indicated that under suitable conditions, asparaginase enzyme can be produced commercially by using the agro-waste, wheat straw on large-scale in an economically feasible way.

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1 Introduction

Over the past few decades, cost-effective agro-waste materials are being utilised in several bioprocess technologies for the production of many value-added products of industrial importance. Some of the most commonly used agro-waste are oil cakes, fruit and vegetable peel, bagasse etc. (Pandey et al., 1999; Ellaiah et al., 2002). Transforming these nutritional rich waste materials into useful bio-products by fermentation process not only minimises the process cost but also reduces the risk of environmental threats (Pandey et al., 1999).

Wheat straw is one of the predominant agro-waste materials with an immense potential due to its wide availability and low-cost. It is an abundant by-product from harvesting of wheat crop in many nations like India. Further, wheat straw has been successfully, used as a raw material for pulp and paper production (Nasser et al., 2015), as a substrate for renewable energy sources such as biogas and bio-ethanol (Ferreira et al., 2014; Huang et al., 2017; Tomás-Pejo et al., 2017), enzymes production (Gao et al., 2008; Ahmed et al, 2018; Shahryari et al., 2018) and also in the commercial cultivation of mushrooms. Even though wheat straw is nutritionally rich, but high lignocelluloses and other nutritional elements are preventing it as a primary feed source for ruminants.

L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is a vital enzyme, involved in the hydrolysis of L-asparagine. It has gained eminent significance due to its potent therapeutic applications especially in treating various forms of leukaemia (Adamson & Fabro, 1968; Umesh et al., 2007; Anjana et al., 2018). The activity of asparaginase is broadly present in plants, animals, and micro organisms. Microbial production of this enzyme has attracted more attention because of its cost-effective and eco-friendly process. Normally, submerged fermentation (SmF) process has been widely used for the production of asparaginase (Basha et al., 2009; Amena et al., 2010; Gurunathan & Sahadevan, 2012). Although, SmF process is a very well established, but it has few drawbacks such as huge volume of waste water generation and difficulties in effluent treatment process (Datar, 1986). To overcome the above disadvantages, solid-state fermentation (SSF) has come into existence as an alternate economical process for the synthesis of various metabolites by utilizing the agro-waste materials (Pandey et al., 1999). SSF has been employed for production of various microbial metabolites (Sircar et al., 1998; Sarada & Sridhar, 1998; Corona et al., 2005; Sandhya et al., 2005; Rojan et al., 2006). As per the documented literature, a wide range of microbial genera such as filamentous fungi (Mishra, 2006; Baskar & Renganathan, 2011), bacteria (Heinemann & Howard, 1969; Abdel-Fattah & Olama, 2002; Kumar et al., 2010; Seyedehe et al., 2011), yeast (Kil et al., 1995; Ramakrishnan & Joseph, 1996) and actinomycetes (Basha et al., 2009) have been reported for the production of asparaginase.

The aim of this study was to synthesize asparaginase enzyme from an inexpensive substrate, wheat straw and optimizing the process parameters that enhance the enzyme productivity. Till today, there is no published data are available related to the production of asparaginase using wheat straw under solid-state fermentation by Fusarium oxysporum NCIM 1008.

2 Materials and Methods

2.1 Materials

Wheat straw was obtained from the nearby agricultural fields of National Capital Region (NCR) area, India. Before use it was cleaned and dried in hot air oven at 60 °C for 24 h, milled and sieved to 1mm particle size. All the chemicals used in this research work were of analytical grade and purchased from Sigma-Aldrich, Bangalore, India.

2.2 Microorganism

The microbial strain, F. oxysporum NCIM 1008 was received from National Centre for Industrial Microorganisms (NCIM), Pune. Obtained culture was maintained on potato dextrose agar (PDA) medium slants at 28 °C for seven days. The slants were stored at 4 °C and were sub-cultured monthly. Under aseptic conditions, F. oxysporum conidial suspension was prepared from a freshly raised seven day old culture by suspending in 10 ml of 0.85% sterile saline solution. This suspension was used as inoculum for subsequent fermentation experiments.

2.3 Solid-state fermentation of wheat straw

Wheat straw (5g) was dispensed into 250ml of cotton-plugged erlenmeyer flasks, moistened with 5 ml of salt solution containing glucose (0.6%), KH₂PO₄ (0.1%), MgSO₄·7H₂O (0.05%) and KCl (0.05%) and autoclaved. Aseptically, the flasks were inoculated with 2 ml of the fungal conidial suspension. The contents in the flasks were mixed uniformly and incubated in a static incubator at 28 °C for about one week (fermentation time) respectively.

2.4 Enzyme extraction and assay

Crude enzyme was extracted as reported by Ghosh et al. (2013). L-asparaginase activity was determined by measuring the amount of ammonia released by Nesslerization according to the method (Wriston & Yellin, 1973). One unit (U) of L-asparaginase is defined as the amount of enzyme required to liberate one μmol of ammonia under optimal assay conditions, and expressed as activity of asparaginase (U) obtained per grams of dry substrate (U/gds).
2.5 Optimization studies

Various crucial process parameters in solid-state fermentation such as fermentation time, initial moisture content, initial pH, incubation temperature and supplementation of nutritional (both carbon and nitrogen) sources were optimised using single-parameter optimization methodology. Samples were drawn continuously at 12 h time interval and the enzyme assay was carried out to calculate the enzyme activity. All the experiments and assays were run in triplicate and the mean values are noted for better results.

3 Results and discussion

The selection of a promising substrate in solid-state fermentation is an essential factor because the production cost of any bioproduct mainly depends upon the cost and availability of the substrate utilized. In this study, wheat straw has been selected as a potential substrate for the production of asparaginase based on its chemical and nutritional composition, cost and availability (Binod et al., 2010; Martin et al., 2012). Further optimization was carried out using the wheat straw as substrate to elevate the enzyme activity.

3.1 Effect of fermentation time

To estimate the fermentation time, SSF was performed with various fermentation time schedules ranging from 12-168 h. For this, wheat straw (5g), inoculated with 2 ml of fungal conidial suspension with initial moisture content of 60 % (v/w) and incubated at 28 °C. Samples were removed for every 12 h and enzyme activity was analysed. The enzyme productivity has shown growth relatedness with the incubation time progressed and maximum enzyme activity (7.04 U/gds) was observed after 96 h (Figure 1). Normally, microbial cell growth and enzyme production were dependent on the duration of fermentation. After 96 h, the enzyme productivity started to decrease gradually. The reason for this is that the microorganism might have reached a stage, from which it could no longer balance its steady growth with the available nutrient resources. The results obtained are in accordance with the data published for the production of L-asparaginase using coconut oil cake (Ghosh et al., 2013).

3.2 Initial moisture content

In SSF, optimum level of initial moisture content is very important parameter and directly affects the maximum substrate utilization, microbial growth and enzyme productivity. Various moisture contents varying from 40-80% (v/w) were taken for SSF. Maximum enzyme activity (9.72 U/gds) was noticed at 60% moisture content (Figure 2) after 96 h of fermentation. Decrease in enzyme activity was noticed at low and high level of moisture content. With increasing moisture content, there is a reduction in porosity and increases the chances of contamination (Lonsane et al., 1985).

3.3 Effect of initial pH of the substrate

In these experiments, the pH of the moistening solution used was set from 4-10 using 1N HCl or NaOH. From the results (Figure 3), maximum asparaginase productivity was noted with pH 6.0 (12.18 U/gds). In view of the data reported, agro-industrial materials used in SSF possess excellent buffering capacity (Pandey & Radhakrishnan, 1992). pH of the medium strongly affects the growth and activity of the micro-organisms. Generally, fungal strains are noted for their best performance in the range of 3.5-6 and low pH avoids contamination by other microbes. Further increase in pH resulted in the reduction of enzyme activity which might be due to the denaturation or inactivation of the microbial strain at extreme acidic and basic pH values.
3.4 Effect of temperature

SSF was carried out at different incubation temperatures varying from 26 to 40 °C. The initial moisture content was maintained at 60 % (v/w), inoculated with 2 ml of the fungal suspension. After 96 h, the samples were extracted and examined for enzyme activity. The fungal strain has shown better growth and enzyme productivity at 30 °C and it was 15.94 U/gds (Figure 4). In biological processes, the temperature is useful in determining the effects of protein denaturation, enzyme inhibition, promotion or suppression of a particular metabolite, cell viability and death. During SSF, there is a general rise in the temperature of the fermenting mass due to respiration (Pandey & Radhakrishnan, 1992).

3.5 Effect of additional nutritional sources

Various available carbon sources (fructose, galactose, glucose, maltose and soluble starch) and nitrogen sources (ammonium sulphate, ammonium chloride, yeast extract, peptone, urea, malt extract, beef extract and L-asparagine) were incorporated to the production medium at 1%, but only L-asparagine has shown a positive impact on enzyme activity and microbial growth to great extent. Since addition of L-asparagine stimulated asparaginase synthesis, attempts were made to estimate the optimum concentration of L-asparagine for maximum enzyme synthesis by the fungal culture. SSF was performed with different doses of L-asparagine varying from 0.25 to 3% (w/w). Enzyme productivity increased with L-asparagine concentration in a relative manner. The maximum enzyme activity (21.54 U/gds) was noticed with 0.75 % L-asparagine dose (Figure 5). With further increase in L-asparagine concentration, there was a gradual decrease in the enzyme yield which might be due to the inhibitory effect of L-asparagine at higher doses on the microbial growth and enzyme productivity.

After optimizing all the essential influential process parameters, final SSF experiment was carried out by incorporating all the standardized optimized parameters and samples were analysed for enzyme productivity. The maximum enzyme yield of 21.54±0.04 U/gds was reported.

Conclusion

Solid-state fermentation was favourably employed to optimize process variables for maximal asparaginase productivity. Under the observed conditions, asparaginase activity of 21.54 U/gds was reported. The results of this investigation have demonstrated that effective utilization of wheat straw might reduce the production cost of therapeutically important asparaginase enzyme.

Acknowledgments

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Conflict of interest

The corresponding author declares that there is no conflict of interest.

References


ASSESSMENT OF CULTURABLE MICROBIAL DIVERSITY OF DHOLELA THERMAL SPRINGS OF GUJARAT, INDIA

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ABSTRACT

The present study deals with bacterial diversity study based on cultivation of the organisms from the Dholela hot springs of Gujarat, India during various seasons. Isolation was carried out by spread plate and streak plate method. Isolates were characterized based on colony and morphological characteristics. Various biochemical tests and 16S rRNA gene analysis were performed for identification of thermophilic bacteria. A total of 18 isolates were cultivated using different types of media. Out of the total 18 isolates, eight were Gram-positive and ten were Gram-negative. Based on the biochemical test of these isolates, 8 were found to produce catalase, 18, 18 and 8 isolates utilized casein, starch, and citrate respectively. Further, 9 isolates were produced H2S by sulphate reduction. Sugar utilization varied in all the isolates. Diversity of the isolates were studied in terms of Simpson, Shannon, Menhinick, Margalef, Berger-Parker and Chao-1 based on the metabolic activity of thermophilic bacteria. Six well-isolated purified colonies were selected for 16S rRNA gene sequence analysis. Based on the molecular identification, these isolated bacterial isolates belonged to genera Bacillus, Brevibacillus, Brevibacterium and Tepidomonas. All these thermophilic isolates may have valuable application at an industrial level and for biotechnological purposes.

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KEYWORDS
Hot spring  
Thermophilic bacteria  
Physicochemical analysis  
Biochemical analysis  
Ecological indices  
16S rRNA gene sequencing

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1 Introduction

Hot springs diversity have been studied worldwide, which are reported as a normal source of several thermophilic bacteria. Geothermal springs are significant spots of microbial biodiversity, having the capacity to produce various enzymes for industrial, agriculture and medical research processes and are also helpful to get novel genes as well as molecules (Saxena et al., 2016; Sahay et al., 2017). Thermophiles are heat-loving microbes, therefore they can grow at 45°C to 75°C with optimum temperature range between 50°C and 60°C. Some of the thermophilic microorganisms tolerate a higher temperature for their survival and growth, whereas other thermophiles tolerate a comparatively lower temperature (Hartmann et al., 1989; Pelczar et al., 1993; Panikov et al., 2003; Eze et al., 2011). The special attraction of hot water springs is the ecological variation of different types of microorganisms and their molecular characteristics (Adiguzel et al., 2011; Genc et al., 2015). Thermophilic bacteria represent both Gram-positive and Gram-negative group and they also belong to aerobic and anaerobic groups of bacteria. Due to their various valuable applications, researchers have focused their research to find out new genus and species of microorganisms around the world (Yoneda et al., 2013; Cihan et al., 2014; Aanniz et al., 2015).

There are several hot springs available in Gujarat. Some studies have been carried out on thermophilic bacteria in TuvaTimba, Lasundra, Tulshisyam, Dholera and Unai (Kikani & Singh 2011; Chudasama 2012; Joshi et al., 2013; Gohel et al., 2013; Dudhagara et al., 2014). Present study was conducted at Dholera hot water spring and focus on seasonal variations of thermophilic organism’s isolates from this ecosystem. Information regarding the microbial community of Dholera hot spring is available in scarcity. The aim of present study was to characterize the thermophilic bacteria from Dholera hot spring in three different seasons and the interest was to find out novel species at this site.

2 Materials and Methods

2.1 Sample collection

Hot spring water samples were collected from Dholera hot spring (22°15’0”N 72°11’27”E), Gujarat. Dholera is an ancient port-city in Gulf of Khambhat, 30 km from Dhabhuka city of Ahmedabad district, Gujarat. Water samples were collected in sterile glass bottles from Dholera hot water spring in three different seasons: monsoon (September), winter (January) and summer (April). Samples were transported in a thermocol box to the laboratory and processed on the same day of the sampling and then stored at room temperature.

2.2 Physicochemical analysis

Temperature and pH of collected samples were recorded at the time of sampling using portable pH meter. Conductivity, TDS and salt of the collected samples were measured in the laboratory using Oakton Thermo, Multi-parameter PCS Testr 35, and Thermo Fisher Scientific.

2.3 Isolation of bacteria

Samples were processed for isolation of bacteria on the same day of the sample collection. Thermophilic microorganisms were isolated directly from water samples by serial dilution technique using various media like Nutrient agar medium (0.5% Peptone, 0.3% Beef extract, 0.5% NaCl, 2.0% Agar), Luria-Bertani medium (1.0% Tryptone, 1.0% NaCl, 0.5% yeast extract, 2.0% Agar), ATCC Thermus medium (0.5% NaCl, 0.5% Peptone, 0.4% Beef extract, 0.2% Yeast extract, 2.0% Agar), Starkey’s sulphate reducing medium (Himedia M1981), Sulphate reducing medium (Himedia M803) and Medium77 medium (0.5% K2HPO4, 1.0% NH4Cl, 0.1% CaCl2·2H2O, 0.1% MgSO4·7H2O, 5.0% Sodium lactate, 1.0% Yeast extract, 5.0% FeSO4·7H2O, 1.0% Sodium thioglycolate, 1.0% Ascorbic acid, 2.0% Agar (Patil et al., 2014). All the plates were incubated at 45 ± 2°C and 50 ± 2°C. After the growth, morphologically distinct colonies were selected and subcultured on respective medium to get pure isolates. Then from these well isolated purified colonies, 20% glycerol stocks of bacterial cultures were prepared for preservation and further study on the isolates.

2.4 The conventional method for identification and characterization of the isolates

The selected isolates were observed for morphological and growth characteristics by the conventional methods as outline below. The bacterial isolates were characterized by Gram staining and observed under light microscope. The thermophilic isolates were identified by conventional, physiological methods and characterised based on biochemical identification tests. These tests are characterized based on Gram nature, shape, temperature, pH, catalase production test, casein utilization test, starch utilization test, citrate utilization test, Sugars profile like D-Glucose, D-Fructose, Maltose, Mannose, Sucrose, Mannitol, Lactose, Xylose, Galactose, H2S production test and Sulphate reduction test as per the Bergy’s manual (Hensyl, 1994).

2.5 Diversity indices

A range of diversity indices profile, cluster analysis and similarity in diversity were generated using statistical software Paleontological Statistics (PAST) considering the results of biochemical tests (Hammer et al., 2001).
2.6 Genomic DNA isolation

Bacterial broth cultures (2.5 ml) were grown in log phase at 45 ± 2 °C and 50 ± 2°C for 24 hours. Overnight cultures (2.0 ml) were centrifuged at 10,000 – 15,000 X g for 15 minutes. The supernatant was decanted and pellet was collected and re-suspended in 1 ml of TE buffer. The DNA was quantified by NanoDrop spectrophotometer by taking absorbance at 260 and 280 nm (Nucleic acid software). The size of DNA was estimated by agarose gel (0.5% W/V) electrophoresis subsequent staining with ethidium bromide and visualisation in U.V. illumination by Gel Documentation System (Gene snap software). Genomic DNA was isolated from the obtained pallets from pure cultures using the miniprep method described by Wilson with some modifications (Moore et al., 2004; Wilson, 1987). The obtained DNA pellets stored at 4 °C for further analysis. 16S rRNA gene sequence analysis was done at Chromous Biotech (Bangalore).

3 Results

3.1 Physicochemical characteristics

Physicochemical characteristics of water samples of Dholera hot spring are listed in Table 1. Dholera water spring was covered with cement concrete construction, hence the soil sample was not available. The temperature of the water was 40° C to 50° C in Dholera. The TDS (Total Dissolved Salt) value varied from 3.1 to 3.7 ppt (parts per thousand) in Dholera. Observed pH value was 7.1 to 7.9 at Dholera sampling point. Conductivity of the studied water samples were reported between 4.4 to 4.9 mS (milli-Siemens). Whereas seasonal physicochemical variations were concerned, the temperature in summer was at high of 50 ° C and at low of 40 ° C in winter, which is significant. Overall seasonal differences in conductivity, TDS and salt concentration in Dholera samples were in the range of 10%.

3.2 Microbial analysis

Total 18 morphologically different isolates were isolated and purified from the water samples collected from Dholera hot springs during summer, monsoon and winter season using Nutrient agar medium, Luria-Bertani medium, ATCC Thermus medium, Starky’s medium, Sulphate reducing medium and Medium77 medium. Out of these isolates; eight isolates were Gram-positive while rest ten were Gram-negative. A wide range of pigmented colonies was observed as shown in table 2. The colours of the pigmented colonies observed were distributed over cream, brown, light brown, black and yellow.

3.3 Metabolic activity based on the biochemical test

Various metabolic activities were assessed to have a broad view of the characterization of bacteria, on the behalf of these isolated strains were identified. Out of 18 isolated isolates, catalase

| Table 1 Morphological observation of all isolates of Dholera hot springs |
|-----------------|-----------------|-----------------|
| Isolates        | Colony observation | Gram nature    |
| DW49            | Cream            | Gram-positive   |
| DW50            | Cream            | Gram-positive   |
| DW51            | Brown            | Gram positive   |
| DW52            | Brown            | Gram-negative   |
| DW53            | Brown            | Gram-negative   |
| DW54            | Blackish         | Gram-negative   |
| DW55            | Cream            | Gram-positive   |
| DW56            | Light brown      | Gram-positive   |
| DW57            | Yellow           | Gram-positive   |
| DW58            | Brown            | Gram-negative   |
| DW59            | Brown            | Gram-negative   |
| DW60            | Blackish         | Gram-negative   |
| DW61            | Cream            | Gram-negative   |
| DW62            | Cream            | Gram-positive   |
| DW63            | Light brown      | Gram-positive   |
| DW64            | Brown            | Gram-negative   |
| DW65            | Brown            | Gram-negative   |
| DW66            | Blackish         | Gram-negative   |

| Table 2 Season-wise physicochemical characteristics observed in samples from Dholera |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Summer          | Monsoon         | Winter          |
| Temperature                    | 50 °C           | 45 °C           | 40 °C           |
| pH                              | 7.9             | 7.2             | 7.1             |
| Conductivity                   | 4.9 ms          | 4.5 ms          | 4.4 ms          |
| TDS                            | 3.7 ppt         | 3.4 ppt         | 3.1 ppt         |
| Salt                            | 2.42 ppt        | 2.38 ppt        | 2.38 ppt        |

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production was showed by 8 isolates. Further, 18, 18 and 9 isolates were able to utilize casein, starch and citrate respectively. H$_2$S production and sulphate reduction properties were reported in nine isolates while sugars metabolism activity varied in all the isolates.

The results of the water samples are summarized in Figure 1. Catalase production was dominant in summer and monsoon seasons isolates, while it was indicated significantly lower in winter season’s isolates. On the other hand, all the three seasons isolates showed positive results for the casein and starch utilization. Citrate utilization was dominant in all three seasons. Sugars profile like fructose, maltose and glucose were positively utilized in all Dholera summer season’s isolates. Rest of the monsoon season’s isolates indicated positive results for utilization of fructose and glucose. Galactose and glucose were also positive in all isolates of winter season’s samples. In the summer and winter season’s isolates, xylose showed positive results in only one isolate. H$_2$S production and Sulphate reduction were dominant in all three seasons.

3.4 Cluster analysis and diversity indices based on metabolic activity

Diversity study of various isolates was carried out in terms of 15 biochemical tests on the basis of their results dendrograms were prepared, and are shown in Figure 2. The blue colour label indicates summer season, green colour indicates the monsoon season and pink colour indicates winter season. All the isolates obtained from water samples collected from Dholera were divided into 2 major group as A and B (Figure 2). B group showed sulphate reducing isolates in all three seasons based on their biochemical results.
Morphologically distinct isolates from Dholera thermal ecosystem were studied to determine their similarity. From Dholera samples total 18 colonies were selected from water samples. Irrespective of the seasonal collection distinct isolates range from 6 to 9 in each sample (Data not shown). Among the different isolates studied the maximum similarity between any 2 isolates was 86 - 87 per cent. Except for DW – 49 and DW – 50 which showed 100 per cent similarity and they were identified as *Bacillus subtilis* based on 16S rRNA partial gene sequence analysis.

Diversity indices data in terms of Taxa_S, Individuals, Dominance, Simpson, Shannon, Menhinick, Margalef, Berger-Parker and Chao-1 were also studied for all the samples collected in the different seasons from the Dholera hot spring ecosystem (Table 3).

### 3.5 Molecular identification of selected organisms

From the total 18 isolates 6 colonies were selected randomly and these isolates were identified based on 16S rRNA gene sequence analysis and results are shown in Table 5. Among the 6 identified cultures three belong to *Bacillus* genus, one to *Brevibacillus*, one to *Brevibacterium* and one to *Tepidimonas*. The details of NCBI identification numbers are given in Table 5.

#### Table 3 Comparison of selected diversity indices of isolates from both ecosystem during sampling season

<table>
<thead>
<tr>
<th>Index</th>
<th>Summer</th>
<th>Dholera Water Monsoon</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxa_S</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Individuals</td>
<td>18-21</td>
<td>18-21</td>
<td>18-23</td>
</tr>
<tr>
<td>Dominance</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Simpson</td>
<td>0.92</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>Shannon</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Menhinick</td>
<td>3.2-3.5</td>
<td>3.2 – 3.5</td>
<td>3.1 – 3.5</td>
</tr>
<tr>
<td>Margalef</td>
<td>4.5 – 4.8</td>
<td>4.5 – 4.8</td>
<td>4.4 – 4.8</td>
</tr>
<tr>
<td>Berger-Parker</td>
<td>0.09-0.1</td>
<td>0.09-0.1</td>
<td>0.08-0.1</td>
</tr>
<tr>
<td>Chao-1</td>
<td>20.1-31.5</td>
<td>20.1-31.5</td>
<td>17.3-31.5</td>
</tr>
</tbody>
</table>

#### Table 4 Detail of NCBI identification

<table>
<thead>
<tr>
<th>Sequence number</th>
<th>Isolates name</th>
<th>Accession number</th>
<th>Isolates number</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRF14</td>
<td><em>Brevibacterium halotolerans</em></td>
<td>MH426315</td>
<td>DW56</td>
</tr>
<tr>
<td>XRF21</td>
<td><em>Tepidimonas Taiwanes</em></td>
<td>MH426322</td>
<td>DW61</td>
</tr>
<tr>
<td>XRF23</td>
<td><em>Brevibacillus brostelensis</em></td>
<td>MH426324</td>
<td>DW55</td>
</tr>
<tr>
<td>XRF24</td>
<td><em>Bacillus subtilis</em></td>
<td>MH426325</td>
<td>DW50</td>
</tr>
<tr>
<td>XRF31</td>
<td><em>Bacillus sonorensis</em></td>
<td>MH614333</td>
<td>DW57</td>
</tr>
<tr>
<td>XRF32</td>
<td><em>Bacillus subtilis</em></td>
<td>MH614334</td>
<td>DW49</td>
</tr>
</tbody>
</table>

4 Discussion and Conclusion

In some hot springs, the physicochemical parameters have been observed to vary with the seasonal effect. While in other studies it has been reported that, the physicochemical parameters can be stable with no significant changes in seasonal effect. Moreover seasonal variation in microbial diversity is low in hot springs due to the high temperature of the springs (Ferris & Ward, 1997; Mackenzie et al., 2013; Briggs et al., 2014; Prieto-Barajas et al., 2017). The prevalent conditions in hot springs such as high water temperature, pH, sulphur concentration, salinity, conductivity and other physicochemical parameters are known to permit the development of selected bacterial communities (Nayak, 2013). In the current study, biochemical tests determined the metabolic activity of the bacterial cells (Goh et al., 2012; Nayak, 2013). Diversity indices tools have been used for the population of bacterial diversity, species richness and also better understanding about microbial community (Kim et al., 2017). Kikani et al. (2015), calculated the various diversity indices of Tulsi Shyam hot spring, Gujarat. They have given information about the evenness in their distribution, community structure and species richness (Kikani et al., 2015). 16S rRNA gene sequencing techniques have been used for the identification of bacteria and phylogenetic
Assessment of culturable microbial diversity of Dholera thermal springs of Gujarat, India

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analysis as the "gold standard" (Ludwig & Schleifer 1999). It has been reported that molecular-based identification of thermophilic bacteria by 16S rRNA gene sequencing is useful at the genus level identification (Sahin et al., 2009). It has earlier been reported that some thermophilic Bacillus species are a source for useful enzymes for industrial and commercial purposes (Rahman et al., 1994; Rao et al., 1998; Dudhagara et al., 2014). In previously published reports, Bacillus spp from the genus Bacillus have been claimed to be largest and the best studied genus (De Boer et al., 1994; Kolsto et al., 2009; Nayak, 2013). Species of genus Bacillus have been found in Lasundra, TuvaTimba, Unai hot springs of Gujarat and these bacillus species have been used for various applications like production of enzyme, biosurfactant, and thermostability etc (Kikani & Singh, 2011; Joshi et al., 2013; Gohel et al., 2013; Dudhagara et al., 2014). According to Hadad et al. (2005) thermophilic bacterium Brevibacillus brostelensis isolated from soil, and this strain plays an important role in biodegradation of polythene (Hadad et al., 2005). B. brostelensis, thermophilic bacteria has been reported from Saudi Arabia hot spring and possesses enzyme having ability to degrade polythene substrates (Khalil et al., 2018). Tepidimonas taiwanensis strain was first reported in a Southern Taiwan hot water spring and it was claimed to be a novel species. Among the Tepidimonas genus, this strain was able to produce alkaline protease enzyme for industrial application (Chen et al., 2006).

The focus of this preliminary study was a comparison of the seasonal diversity of Dholera hot spring. Further present investigation also focused on the identification of seasonal diversity to get novel species. Based on bacterial identification study, almost similar numbers of isolates were found in all seasons of Dholera hot spring. 16S rRNA sequence-based molecular identification shows a good amount of correlation with the biochemical identification of thermophilic bacteria. The presence of T. taiwanensis species is reported for the first time in the Dholera hot springs of Gujarat. Based on our morphological and biochemical studies the isolates divided in two major groups such as sulphate reducers and sulphate non-reducers.

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Conflict of interest

Author declares that there is no conflict of interest.

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Genc B, Nadaroglu H, Adiguzel A, Baltaci O (2015) Purification and characterization of an extracellular cellulase from...


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ANTIDIABETIC POTENTIAL OF Costus igneus LEAF IN STREPTOZOTOCIN INDUCED DIABETIC WISTAR ALBINO RATS

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KEYWORDS
Costus igneus
Streptozotocin
Glibenclamide
Blood urea nitrogen

ABSTRACT

Traditional herbal medicinal plants leaves essentially reduced the fasting and postprandial blood sugar levels and bringing them down towards normal. The present study was aimed to evaluate acute toxicity, in vivo anti-diabetic effect of C. igneus on streptozotocin induced diabetic wistar albino rats by estimating total cholesterol, triglycerides, high density lipoprotein, low density lipoprotein, very low density lipoprotein cholesterol, serum creatinine and blood urea nitrogen. The result of acute toxicity study revealed that various tested extracts did not show any mortality at all tested concentration. Further, hexane extract of C. igneus leaves at 200mg showed significant (P<0.001) reduction in blood sugar level (114.8± 7.08) while in case of 400mg hexane leaf extract this reduction in sugar level (91.6± 6.12) was started from day 7th to 28th day. Further, the same treatment also showed highly significant (P<0.001) reduction in blood urea nitrogen (30.52±1.42) and it was equipotent as that of Glibenclamide. Similarly significant reduction in serum creatinine level was observed at 400 mg/kg in all the three leaf extracts of C. igneus and was similar to the effect produced by that of Glibenclamide. The present study explored the significant anti-diabetic potential of C. igneus and from the results of the study it can be concluded that C. igneus can be used as safe and cost-effective herbal drug for the treatment of diabetes.

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1 Introduction

Diabetes is a metabolic disorder of endocrine system that participate disturbance in glucose, lipid and protein homoeostasis (Van et al., 2006). Diabetes is generally characterized by hyperglycemia, glucosuria, polyuria and loss of body weight (Tende et al., 2011). Various allopathic medicines are available for the treatment of diabetes but these have various side effects. Traditional herbal medicinal plants have been used widely to effectively treat diabetes (Mukherjee et al., 2006), these herbal products are safer than synthetic formulation (Abou & Ghanema, 2013). Plants which have hypoglycemic action act on blood glucose through several mechanisms. Some of them may inhibit endogenous glucose production (Eddouks et al., 2003) or interfere the gastrointestinal glucose absorption (Musabayane et al., 2006), some may produce insulin like substance (Collier et al., 1987; Gray&Flatt,1999) some may inhibit insulinase activity and some may enhance secretion of insulin from β cells of the pancreas (Khan et al., 1990; Trivedi et al., 2004; Yadav et al., 2008). While some plants may proliferate β cells in pancreas by activating regeneration of these cell (Shanmugasundaram et al., 1990). In diabetes, free radicals are adversely formed through glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins (Maritim et al., 2003; Mehta et al., 2006). Cancers, diabetes, heart and brain related disease are mainly caused by production of free radicals which alter the structural and functional cell components in human beings (Mclarty, 1997; Yang et al., 2001; Young & Wood, 2001; Sun et al., 2002; Bimal et al., 2011). Large number of plants reputed to possess anti-diabetic properties (Saraswat et al., 2010; Ugwuju et al., 2010; Jafri et al., 2011). Among these very few have received equitable scientific and medical scrutiny in terms of their anti-glycation activities. The currently available anti-diabetic drugs may have some limitations and side effects therefore the growing trend for diabetes treatment was directed to the use of natural agents, such as medicinal herbs (Awanish et al., 2011). Therefore present study was aimed to evaluate acute toxicity, in-vivo anti-diabetic effect of C. igneus on streptozotocin induced diabetic wistar albino rats and determination of various biochemical parameters.

2 Materials and methods

2.1 Collection and identification of plant material

The leaves of C. igneus were collected from the outskirts of Hosur, Krishnagiri district of Tamil Nadu and identified on the behalf of taxonomy key developed by Dr. M. Kumar, Assistant Professor, Department of Plant Biology and Biotechnology at Madras Christian College, Chennai Tamil Nadu India. The collected leaf materials were cleaned shade dried and powdered for further extraction.

2.2 Preparation of extracts

The air dried leaf powdered of C. igneus were extracted in Soxhlet extractor for 8-10 hrs, successively with acetone, hexane and hot water and the air dried residues were further extracted with hot water by the method of maceration for 24 hrs and the material was dried in hot air oven at 40°C. The evaporated extracts thus obtained were dissolved in the respective solvents at the concentration of 1 mg/mL and used for further in vivo studies.

2.3 Animals and Management

The experiments were carried out using Wistar albino rats (150–200 g) procured from the Animal house, Nandha college of Pharmacy, Erode, Tamilnadu, India. On arrival the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24 ± 2°C and relative humidity of 30–70 %. A 12:12 light:dark cycle was followed. All animals were allowed to free access to water and fed with standard commercial rat chaw pallets (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics Committee (Regd. no: 688/PO/Re/S/202/CPCSEA) and were in accordance with the guidelines of the CPCSEA.

2.4 Acute toxicity study

Acute toxicity studies were performed according to OECD-423 (Organization of Economic and Cooperation Development) guidelines. Male Swiss mice were selected by random sampling technique for this study. The animals (n=5) were fasted for 4 hrs with free access to water. The different leaf extracts of C. igneus was administered orally at a dose of 5 mg/kg initially and mortality if any was observed for 3 days. If any mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then higher (50, 300, 2000 mg/kg) doses of the different leaf extracts of C. igneus were employed for further toxicity studies. The following general behaviour sedative, hypnotics, convulsion, ptosis, analgesia, stupar reaction, motor activity, muscle relaxant, pilo erection, change in skin colour, lacrimal secretion, stool consistency was also observed during the acute toxicity study Ecobichon,(1997).

2.5. Experimental induction of diabetes

For inducing the diabetes, all the experimental group animals were kept fasting for overnight. Diabetes was induced by intra-peritoneal injection of streptozotocin (STZ) dissolved in 0.1 M cold sodium citrate buffer, pH 4.5, at a dose of 55 mg/kg (Aslam
et al., 2007). Group one and two served as untreated healthy, untreated streptozotocin diabetic control and received distilled water. To overcome the drug induced hypoglycaemia the animals were allowed to drink 5% glucose solution overnight. A week later the blood glucose level were checked, the rats with blood glucose range of above 200 mg/dl were considered as diabetic rats and used for the experiment.

2.6 Experimental design

For evaluating the hypoglycemic effect of various extracts of C. igneus all the animals were randomly divided in to 9 groups with 5 animals in Each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Normal control (Non – diabetic) received distilled water 1ml/kg</td>
</tr>
<tr>
<td>Group II</td>
<td>Diabetic (streptozotocin induced) rats received distilled water 1ml/kg</td>
</tr>
<tr>
<td>Group III</td>
<td>Diabetic rats received Standard drug Glibenclamide 5mg/kg</td>
</tr>
<tr>
<td>Group IV</td>
<td>Diabetic rats received 200 mg/kg hot water extract of C. igneus leaf</td>
</tr>
<tr>
<td>Group V</td>
<td>Diabetic rats received 400mg/kg hot water extract of C. igneus leaf</td>
</tr>
<tr>
<td>Group VI</td>
<td>Diabetic rats received 200 mg/kg of acetone extract of C. igneus leaf</td>
</tr>
<tr>
<td>Group VII</td>
<td>Diabetic rats received 400mg/kg of acetone extract of C. igneus leaf</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Diabetic rats received 200 mg/kg of hexane extract of C. igneus leaf</td>
</tr>
<tr>
<td>Group IX</td>
<td>Diabetic rats received 400mg/kg of hexane extract of C. igneus leaf</td>
</tr>
</tbody>
</table>

All the above mentioned experiments were carried out for a period of 28 days. Blood glucose levels were determined on initial (0 day), 4th, 7th, 14th and 28th day. On 29th day the animals under pento-barbitone sodium anesthesia, blood was collected through sinus or retro-orbital puncture and serum was separated for biochemical estimations.

2.7 Determination of serum lipid profile

The blood was collected through sinus or retro-orbital puncture from the animals on the termination day, serum was separated and subjected for analysis of total cholesterol, triglycerides, high density lipoprotein cholesterol, low density lipoprotein, very low density lipoprotein cholesterol, creatinine and blood urea nitrogen.

2.7.1 Total cholesterol

The analysis of total cholesterol in serum was determined by a colorimetric method described by Roeschlau et al. (1974). This assay principally based on enzymatic hydrolysis and oxidation of cholesterol and the indicator compound, quinoneimine is formed from hydrogen peroxide and 4-aminophenazine in the presence of phenol and peroxidase. Composition of reagents involves the mixture of following, 4-aminophenazine (0.03 mmol/l), phenol (6 mmol/l), peroxidase (≥0.5 U/ml), cholesterol esterase (> 0.15 U/ml), cholesterol oxidase (> 0.1 U/ml) and pipes buffer (80 mmol/L pH 6.8). The serum sample (10 μl) was mixed with 1 ml of reagent, incubated at 37°C for 5 min, and absorbance measured at 500 nm against the reagent blank.

2.7.2 Triglycerols

The determination of triglycerides (TG) was carried out by colorimetric method given by Tietz, (1990). Principle of the assay involves enzymatic hydrolysis of TG with lipases and the indicator is a quinoneimine formed from hydrogen-peroxide, 4-aminophenazine and 4-chlorophenol under the catalytic activity of peroxidase The enzyme reagent consisted of 4-aminophenazine (0.5 mmol/l), ATP (1.0 m.mol/l), lipases (≥150 U/ml), glycerol-kinase (≥0.4 U/ml), glycerol-3-phosphate oxidase (≥1.5 U/ml), peroxidase (≥0.5 U/ml). The serum sample (10 μl) was mixed with 1000 μl of enzyme reagent, incubated at 37°C for 5 min and absorbance measured at 500 nm against the reagent blank.

2.7.3 HDL Cholesterol

Serum HDL cholesterol was determined using a colorimetric method described by Lopes et al. (1977). Principally the assay is based on quantitatively precipitation of low density lipoproteins (LDL and VLDL) and chylomicron fraction by the addition of phophotungstic acid in the presence of magnesium ions. The cholesterol concentration in the HDL fraction after centrifugation remains in the supernatant is determined. The precipitation reagents consisted of phosphotungstic acid (0.55mmol/l) and magnesium chloride (25mmol/l). The serum sample (200μl) was mixed with 500 μl of precipitation reagent and centrifuged at 4000 rpm for 10 min. The supernatant (100μl) was incubated at 37°C for 5 min and absorbance measured at 500 nm against the reagent blank. The cholesterol standard was 200 mg/dL (5.17 mmol/l).

The concentration of cholesterol in the supernatant was calculated by following formula

\[
\text{HDL Cholesterol} = \text{ΔA sample} / \text{ΔA standard} \times \text{concentration of standard}
\]

2.7.4 LDL and VLDL Cholesterol

The calculation of low density lipoprotein (LDL) and Very low density lipoprotein (VLDL) was done according to Friedwald formula (Friedewald et al., 1972).

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http://www.jebas.org
LDL = TC – HDL – VLDL,

VLDL cholesterol = Triglycerides / 5

2.7.5 Estimation of Blood Urea

Estimation of blood urea nitrogen was performed according to method given by Natelson et al. (1951). Three test-tubes were taken and labelled as B, T and S. In test tube B 0.02 ml of water was pipetted, in test tube T 0.02 ml of blood was taken and in test tube S 0.02 ml of standard solution of urea (40 mg urea in 100 ml of water) was taken. To all three test tube 0.1 ml of diacetylmonoxime solution and 5 ml of acid reagent (Thiosemicarbazide) was added. The reaction mixture in these test tubes was mixed and kept in a boiling water bath for 15 minutes. After cooling, the absorbance was read at 540 nm and concentration of urea in mg/dl was calculated.

2.7.6 Estimation of Serum Creatinine

The estimation of serum creatinine was performed according to the method described by Slot, (1965). Three test-tubes were taken and labelled as B, T and S. 2 ml of water was taken in test tube B, 2 ml of serum and 4 ml of water was taken in test tube T and in test tube S, 3 ml of water and 1 ml of creatinine was taken. 2 ml of ammonium sulphate and 2 ml of sodium tungstate was added in all the three test-tubes and centrifuged. 3 ml of supernatant was removed from each test tube and to the supernatant 1 ml of picric acid and distilled water was added. Absorbance was read at 520 nm and concentration of serum creatinine in mg/dl was calculated.

2.8 Statistical Analysis

The values of results were expressed as mean ± SEM. The statistical analysis of data was carried out by one way analysis of variance (ANOVA) followed by Dunnet’s ‘t’ test. P values <0.05 were considered significant.

3 Results

3.1 Oral acute toxicity study of various leaf extracts of C. igneus

The results of acute toxicity study of hot water, acetone and hexane extracts of C. igneus leaf were shown in Table 1. All tested leaf extracts of C. igneus has not shown any mortality even after 72 hours at 2000 mg/kg. The acetone and hexane extracts of C. igneus leaves showed mild sedation and hexane extract showed muscle relaxant and CNS depressant activity at 2000 mg/kg. While hot water extract of C. igneus leaves didn’t alter any of the general behaviour. There was no lethality or toxic reported during and after the study period with all the three different leaf extract of C. igneus.

3.2 Hypoglycemic effect of C. igneus leaf extract

The effect of different leaf extracts of C. igneus was studied for its hypoglycemic effect against Streptozotocin induced diabetic rats and the results are shown in Table 2. Glibenclamide was used as reference control and it significantly reduced the blood sugar levels from 4th day onwards and on 28th day the blood sugar was

<p>| Table 1 Oral acute toxicity study of different leaf extracts of C. igneus (2000mg/kg) in mice |
|---------------------------------|---------------------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>S. No.</th>
<th>General Behaviour</th>
<th>Hot Water Extracts</th>
<th>Acetone Extracts</th>
<th>Hexane Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sedation</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Hypnosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Convulsion</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Ptosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Analgesia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Stupar Reaction</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Motor activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Muscle Relaxant</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>CNS Stimulant</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>CNS Depressant</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Pilo Erection</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Skin Colour</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Lacrimation</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Stool Consistancy</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*'+' Present & '-.' Absent
Table 2 Effect of different extracts of C. igneus leaf on blood sugar level in Streptozotocin induced wistar albino rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Sugar Level (mg/dl)</th>
<th>4th day</th>
<th>7th day</th>
<th>14th day</th>
<th>28th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>98±4.66</td>
<td>98±3.12</td>
<td>101±4.71</td>
<td>91.0±3.67</td>
<td>98±3.94</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>100.6±8.12</td>
<td>213.0±9.62</td>
<td>220±8.15</td>
<td>215.2±9.06</td>
<td>215.4±8.92</td>
</tr>
<tr>
<td>Standard Glibenclamide (5mg/kg)</td>
<td>88.0±3.41</td>
<td>217.6±3.72</td>
<td>175.8±3.69</td>
<td>151.6±4.03</td>
<td>129.2±2.92</td>
</tr>
<tr>
<td>Hot water extract 200mg/kg</td>
<td>100.6±9.96</td>
<td>212.2±8.12</td>
<td>196.2±8.06</td>
<td>173.6±7.84</td>
<td>169.2±7.92</td>
</tr>
<tr>
<td>Hot water extract 400mg/kg</td>
<td>97.2±6.12</td>
<td>213.8±8.31</td>
<td>184.2±7.39*</td>
<td>164.8±7.82*</td>
<td>141.8±6.94</td>
</tr>
<tr>
<td>Acetone extract 200mg/kg</td>
<td>98.0±6.51</td>
<td>211.8±7.36</td>
<td>185.6±7.81*</td>
<td>178.0±8.01*</td>
<td>143.8±6.94</td>
</tr>
<tr>
<td>Acetone extract 400mg/kg</td>
<td>96.8±7.02</td>
<td>217.0±8.62</td>
<td>183.4±7.62*</td>
<td>145.4±8.12*</td>
<td>111.6±8.22</td>
</tr>
<tr>
<td>Hexane extract 200mg/kg</td>
<td>106.2±8.02</td>
<td>212.6±7.09</td>
<td>172.6±6.92**</td>
<td>162.4±6.86**</td>
<td>120.8±7.12</td>
</tr>
<tr>
<td>Hexane extract 400mg/kg</td>
<td>95.0±6.12</td>
<td>212.6±7.32</td>
<td>165.4±7.16**</td>
<td>132.8±6.82***</td>
<td>108.8±7.09</td>
</tr>
</tbody>
</table>

Values are in mean ± SEM (n=6), *P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

Table 3 Effect of different extracts of C. igneus leaf on lipid profiles in Streptozotocin induced wistar albino rats

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Total Cholesterol</th>
<th>Triglycerols</th>
<th>HDL - Cholesterol</th>
<th>LDL - Cholesterol</th>
<th>VLDL - Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111.32±5.87</td>
<td>34.83±3.28</td>
<td>42.11±2.63</td>
<td>18.63±1.08</td>
<td></td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>142.80±6.90</td>
<td>31.62±2.31</td>
<td>21.63±1.01</td>
<td>76.25±4.89</td>
<td>31.54±1.76</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>119.01±7.62***</td>
<td>33.57±1.16***</td>
<td>45.16±2.60**</td>
<td>21.14±1.70**</td>
<td></td>
</tr>
<tr>
<td>Hot Water Extract 200 mg</td>
<td>123.72±6.03*</td>
<td>26.63±0.87*</td>
<td>58.18±3.42**</td>
<td>23.59±1.62*</td>
<td></td>
</tr>
<tr>
<td>Hot Water Extract 400 mg</td>
<td>117.74±4.42**</td>
<td>32.17±1.73***</td>
<td>45.05±2.97**</td>
<td>22.68±1.71**</td>
<td></td>
</tr>
<tr>
<td>Acetone Extract 200 mg</td>
<td>123.90±5.20*</td>
<td>25.63±1.92*</td>
<td>61.35±4.42*</td>
<td>24.50±1.62*</td>
<td></td>
</tr>
<tr>
<td>Acetone Extract 400 mg</td>
<td>116.22±4.40**</td>
<td>31.47±2.46***</td>
<td>43.06±1.97**</td>
<td>21.67±1.71**</td>
<td></td>
</tr>
<tr>
<td>Hexane Extract 200 mg</td>
<td>127.15±5.21*</td>
<td>25.82±1.02*</td>
<td>57.32±2.42**</td>
<td>24.51±1.62*</td>
<td></td>
</tr>
<tr>
<td>Hexane Extract 400 mg</td>
<td>118.62±3.19**</td>
<td>31.61±1.94***</td>
<td>45.60±3.97***</td>
<td>20.66±1.71**</td>
<td></td>
</tr>
</tbody>
</table>

Values are in mean ± SEM (n=6), *P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

found 119.8±3.16 mg/dl. Among various tested extract, 200 mg/kg of hot water extract of C. igneus leaves moderately decreased the blood sugar level (142.8±8.02) from 4th day onwards and the effect was maintained until the end of the treatment against the reference control 119.8±3.16mg/dl. As compared to other extracts, hexane extracts C. igneus at 400mg/kg has shown high significant (P<0.001) decrease in blood sugar levels (91.6±6.12) form 7th day to 28th day of treatment as that of the reference control 119.8±3.16 mg/dl.

3.3 Effect of leaf extracts of C. igneus on Lipid Profile

The results of analysis of Total cholesterol (TC), Triglycerides (TG), High Density Lipoprotein Cholesterol (HDL-C), Low Density Lipoprotein Cholesterol (LDL-C) and Very Low Density Lipoprotein Cholesterol (VLDL-C) are given in Table 3.

3.3.1 Total cholesterol

The animals treated with streptozotocin (STZ) have higher value of total cholesterol as compared to normal control. Glibenclamide (5 mg/kg) treatment significantly (P<0.001) reduced the level of total cholesterol (119.01±7.62) which enhanced by STZ treatment (142.80±6.90). The low dose (200 mg/kg) of hot water, acetone and hexane extracts of C. igneus were observed less significant (P<0.05) in reducing cholesterol level as compared to diabetic control. High dose (400 mg/kg) of hot water (117.74±4.42) and acetone leaf (116.22±4.40) extract of C. igneus was found more significant (P<0.01) to decrease cholesterol level (118.62±3.19) as compared to diabetic control (142.80±6.90).
3.3.2 Triglycerols

The animals treated with streptozotocin (STZ) enhanced the triglycerides compared to normal control. Treatment with glibenclamide (5 mg/kg) significantly (P<0.001) decreased the level of triglycerides (113.62±6.31) enhanced by streptozotocin (STZ) (113.62±6.31). The low dose (200 mg/kg) of hot water, acetone and hexane extracts of *C. igneus* were found less significant (P<0.05) to decrease triglycerides. Whereas hot water (72.93±3.76), acetone (73.50±3.17) and hexane (75.36±5.27) extract at 400 mg/kg has shown high significance (P<0.01) reduction in triglycerides level (75.36±5.27) as compared to diabetic control (113.62±6.31).

3.3.3 HDL Cholesterol

The animals treated with streptozotocin (STZ) have elevated level of HDL cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the level of HDL cholesterol (33.57±1.16). Low dose (200 mg/kg) of hot water (26.63±0.87), acetone (25.63±1.92) and hexane (25.82±1.02) extracts of *C. igneus* were found less significant (P<0.05) to decrease the level of HDL cholesterol as compared to diabetic control (21.63±1.01). Whereas high dose (400 mg/kg) of hot water (32.17±1.73), acetone (31.47±2.46) and hexane (31.61±1.94) extract of *C. igneus* were found more significant (P<0.01) in reducing the level of HDL cholesterol as compared to diabetic control (21.63±1.01).

3.3.5 LDL and VLDL Cholesterol

Induction of streptozotocin (STZ) in animals increased the LDL cholesterol level as compared to normal control. Treatment with glibenclamide (5 mg/kg) significantly (P<0.001) reduced the LDL cholesterol level (45.16±2.60) enhanced by streptozotocin (STZ) (76.26±4.89). It was observed that low dose (200 mg/kg) of hot water (58.18±3.42), acetone (61.35±4.42) and hexane (57.32±2.42) extracts of *C. igneus* were less significant (P<0.05) in reducing the LDL cholesterol level as compared to diabetic control (76.26±4.89). The high dose (400 mg/kg) of hot water extracts of *C. igneus* was found least significant (P<0.01) to reduce LDL cholesterol level (45.05±2.97). Similarly high dose (400 mg/kg) of acetone and hexane extracts of *C. igneus* more significantly (P<0.01) reduced the LDL cholesterol level (43.06±1.97), (45.60±3.97) respectively as compared to diabetic control (76.26±4.89). The results of VLDL cholesterol analysis revealed that there was less significant (P<0.05) reduction of VLDL cholesterol levels on treatment with hot water, acetone and hexane leaf extracts of *C. igneus* as compared to diabetic control. High dose (400 mg/kg) of hot water leaf extract *C. igneus* showed more significant (P<0.01) potential in reduction of VLDL cholesterol (22.68±1.71) as compared to diabetic control (31.54±1.76). Similarly high dose of acetone and hexane extracts of *C. igneus* leaves also showed more significant (P<0.01) reduction in VLDL cholesterol (21.67±1.71), (20.66±1.71) respectively as compared to diabetic control (31.54±1.76).

3.3.6 Effect of different leaf extracts on serum blood nitrogen (BUN)

The effects of various leaf extracts of *C. igneus* on serum blood urea nitrogen (BUN) in STZ induced diabetes rats and the results were shown in Table 4. The blood urea nitrogen was increased in the STZ induced diabetes animals as compared to normal control animals. Hot water and acetone leaf extracts of *C. igneus* at dose of 200 mg/kg showed less significance (P<0.05) in bringing down blood urea nitrogen level as compared to diabetic control. Whereas hexane extract at 200 mg/kg found significant (P<0.01) in lower down the blood urea nitrogen (38.17±2.22). At high dose 400 mg/kg among all the three leaf extracts of *C. igneus*, hexane extract showed high significance (P<0.001) in reduction of blood urea nitrogen level (30.52±1.42) as compared to diabetic control (51.82 ± 2.67). It was observed the effect of hexane extract on blood urea nitrogen was equipotent as that of the reference control Glibenclamide.

Table 4 Effect of various leaf extracts of *C. igneus* on Blood urea nitrogen and Creatinine in Streptozotocin induced wistar albino rats

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.32 ± 1.72</td>
<td>0.48 ±0.06</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>51.82 ± 2.67</td>
<td>0.16 ±0.01</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>32.56 ± 1.63***</td>
<td>0.37 ±0.04***</td>
</tr>
<tr>
<td>Hot Water Extract 200 mg</td>
<td>48.73±2.22*</td>
<td>0.29 ±0.01*</td>
</tr>
<tr>
<td>Hot Water Extract 400 mg</td>
<td>31.62 ±5.86***</td>
<td>0.34 ±0.01***</td>
</tr>
<tr>
<td>Acetone Extract 200 mg</td>
<td>45.61±2.09*</td>
<td>0.24±0.01*</td>
</tr>
<tr>
<td>Acetone Extract 400 mg</td>
<td>32.50±1.62***</td>
<td>0.35±0.02***</td>
</tr>
<tr>
<td>Hexane Extract 200 mg</td>
<td>38.17±2.22***</td>
<td>0.29±0.01*</td>
</tr>
<tr>
<td>Hexane Extract 400 mg</td>
<td>30.52±1.42***</td>
<td>0.36±0.01***</td>
</tr>
</tbody>
</table>

Values are in mean ± SEM (n=6),*P<0.05, **P<0.01, ***P<0.001 Vs Diabetic Control

3.3.7 Effect of different leaf extracts on Serum Creatinine

Serum creatinine level was enhanced by the STZ administration and it was reversed by the Glibenclamide. Treatment containing 200 mg/kg dose of *C. igneus* hot water has less significant (P<0.05) effect on reducing creatinine level (0.29±0.01) similar effect has been reported for 200 mg/kg dose of acetone (0.24±0.01) and hexane (0.29±0.01) extract. Further a significant reduction was reported in serum creatinine level at 400 mg/kg in
all the three, hot water (0.34 ±0.01), acetone (0.35±0.02) and hexane (0.36±0.01) leaf extracts of *C. igneus* as compared to normal control (0.48 ±0.06). The effect produced by hot water, acetone and hexane leaf extracts of *C. igneus* at dose of 400 mg/kg was similar to effect produced by that of Glibenclamide (0.37 ±0.04).

4 Discussion

The results of the study revealed that all the extracts of *C. igneus* did not have any mortality at 2000mg/kg even after 72 hours. Although acetone and hexane extracts showed mild sedation while hexane extract showed muscle relaxant and CNS depressant activity at 2000mg/kg. Further hot water extract didn’t alter the general behaviour of experimental animals. No lethality or toxic reactions found during and after the study period with all the three different extract of *C. igneus*. Substances with 50%lethal dose of 1000 mg/kg body weight/oral route are regarded as being safe or of low toxicity as per (Clarke & Clarke, 1977).

Plant derivatives with hypoglycemic properties have been used in folk medicine and traditional healing systems around the world from ancient times. Despite, the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major medical problem to people (Ravi et al., 2005). Medicinal plants used to treat hypoglycemic and hyperglycemic conditions are of considerable interest to ethno-botanical community as the plants contain valuable medicinal properties in its different parts. Study carried by Palanivel et al. (2013) observed that diabetic rats treated with ethanolic extract of *C. igneus* 250mg/kg decreased blood glucose levels at the end treatment which was carried for 14 days indicating good hypoglycemic activity. Treatment with ethanolic and aqueous extracts of *C. igneus* at a dose of 500mg/kg showed significant reduction in increased blood glucose (Kumudhavalli & Jaykar, 2012).

Present study on hypoglycemic effect of *C. igneus* in streptozotocin induced diabetic rats revealed that experimental animals with blood glucose range of above 200 mg/dl were considered as diabetic rats after the administration of STZ. Glibenclamide was used as reference control and it significantly reduced the blood sugar levels. 200mg/kg dose of hexane extract of *C. igneus* showed significant (P<0.001) decline in blood sugar level. Among the higher doses of 400 mg/kg hexane extract of *C. igneus* showed high significant (P<0.001) reduction in blood sugar level form 7th day to 28th day of treatment.

Reports of clinical trials have demonstrated that the increase in plasma low density lipoprotein cholesterol (LDL-C) levels is implicated in the early development and progression of atherosclerosis. West et al. (1983) revealed that in diabetes triglycerides are also a risk and high density lipoprotein cholesterol (HDL-C) is an anti-atherogenic fraction. In normal condition it is well known that lipoprotein lipase enzyme is activated by insulin and hydrolyzes the triglycerides. The reports of previous studies on evaluation of anti-diabetic effect of ethanolic and aqueous extracts of *C.igneus* in streptozotocin induced diabetes. The treatment with ethanolic and aqueous extracts of *C.igneus* at a dose of 500mg/kg for the period of 15 days showed significant reduction in cholesterol, triglycerides, LDL and elevated the decreased HDL level as that of standard (Kumudhavalli & Jaykar, 2012).

The effect of different leaf extracts of *C. igneus* on blood serum sample were subjected to lipid analysis and biochemical parameters like total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) in the present study. The animals treated with streptozotocin (STZ) have higher value of total cholesterol as compared to normal control. Glibenclamide (5 mg/kg) treatment significantly (P<0.001) reduced the level of total cholesterol which enhanced by STZ treatment. The low dose (200 mg/kg) of hot water, acetone and hexane extracts of *C. igneus* were observed less significant (P<0.05) in reducing cholesterol level as compared to diabetic control. The high dose (400 mg/kg) of hot water leaf extract and acetone extract of *C. igneus* was found more significant (P<0.01) to decrease cholesterol respectively as compared to diabetic control. Similarly high dose (400mg/kg) of hexane extracts of *C. igneus* found more significant (P<0.01) to reduce cholesterol level as compared to diabetic control.

The animals treated with streptozotocin (STZ) enhanced the triglycerides compared to normal control. Treatment with glibenclamide (5 mg/kg) significantly (P<0.001) decreased the triglycerides enhanced by streptozotocin (STZ). The low dose (200 mg/kg) of hot water, acetone and hexane extracts of *C. igneus* were find less significant (P<0.05) to decrease triglycerides. Whereas higher dose (400 mg/kg) of hot water extract found significant (P<0.01) in reducing triglyceride level. Similarly high dose (400 mg/kg) of acetone extract was found more significant (P<0.01) to decrease triglycerides. The high dose (400 mg/kg) of hexane extract of *C. igneus* also found more significantly (P<0.01) in reducing triglycerides level as compared to diabetic control.

The animals treated with streptozotocin (STZ) have elevated level of HDL cholesterol compared to normal control. Glibenclamide (5 mg/kg) significantly (P<0.001) decreased the level of HDL cholesterol. Lower dose (200 mg/kg) extracts of *Cigneus* were found less significant (P<0.05) to decrease the level of HDL cholesterol. Whereas higher dose (400 mg/kg) extracts were found
more significant in reducing HDL cholesterol level as compared to diabetic control (P<0.01).

Induction of streptozotocin (STZ) in animals increased the LDL cholesterol and VLDL cholesterol compared to normal control. Treatment with glibenclamide (5 mg/kg) significantly (P<0.001) reduced the LDL cholesterol level enhanced by streptozotocin (STZ). It was observed that all three extracts of C. igneus (200 mg/kg) were less significant (P<0.05) in reducing the LDL cholesterol and VLDL cholesterol levels. However high dose (400 mg/kg) of C. igneus extracts was found more significant (P<0.01) to reduce LDL cholesterol and VLDL cholesterol level.

Previous study by Palanivel et al. (2013) reported that C. igneus ethanolic extract (Whole plant) 250mg/kg also showed statistically significant decrease (p<0.01) in blood urea nitrogen and creatinine levels as compared to diabetic control. The reduction in BUN in animals receiving various plant extracts interpreted as mechanism responsible for reabsorption of urea in nephrons. Creatinine, on other hand is organic base formed of degradation outcome of creatinine phosphate produced in muscle protein metabolism Mayes, (1988).

In the present study the effects of various extracts of C. igneus leaves on serum blood urea nitrogen (BUN) and creatinine in STZ induced diabetes in rats showed that the blood urea nitrogen and serum creatinine was increased in the STZ induced diabetes animals as compared to normal control animals. Treatment with C. igneus hexane extracts have highly significant (P<0.001) effect in reduction of blood urea nitrogen level at both concentrations and its effect on blood urea nitrogen was equipotent as glibenclamide. Similarly the high dose (400 mg/kg) of all the extracts of C. igneus has shown a significant reduction in serum creatinine level(P<0.001).

**Conclusion**

Acute toxicity studies of C. igneus extracts revealed no toxic reactions and did not have any mortality at 2000mg/kg even after 72 hours. Hexane extract of C. igneus shown high significant hypoglycaemic activity in streptozotocin induced diabetic rats and at 400 mg/kg extracts of C. igneus has shown high significance in reduction of serum lipid profile. The effect of hexane extract at 400 mg/kg on blood urea nitrogen and serum creatinine was similar to the effect produced by glibenclamide. The present study explored that C. igneus has potential to reduce the blood glucose level and other factors associated with diabetes. Hence C. igneus can act as natural, safe and cost-effective for the treatment of diabetes.

**Conflict of interest**

The corresponding author declares that there is no conflict of interest.

**Funding sources**

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**Acknowledgment**

I express my sincere and special thanks to Dr. S. Sengottuvelu, Head and Mr. S. HajaSherief, Assistant Professor, Department of Pharmacology, Nandha College of Pharmacy, Erode Tamilnadu India for permitting me to work in his laboratory to carry out the animal model study

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West KM, Ahuja, Bennett PH(1983) The role of circulating glucose and triglyceride concentrations and their interactions with other ‘risk factors’ as determinants of arterial disease in nine diabetic population samples from the WHO multinational study. Diabetes Care 6:361–369.


CAN RUMINANT METABOLIZABLE ENERGY OF BARLEY, CHICKPEA AND LENTIL STRAW BE PREDICTED USING CHEMICAL COMPOSITION?

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ABSTRACT

This study attempted to generate simple and robust models to predict metabolizable energy (ME) content of barley, chickpea and lentil straw using chemical composition. Crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and ME of 1933, 487 and 489 straw samples of barley, chickpea and lentil respectively were determined using near infrared reflectance spectroscopy. The samples belonged to 1933 genotypes of barley, 79 genotypes of chickpea and 66 genotypes of lentil. Barley samples were collected from experimental locations of International Center for Agricultural Research in the Dry Areas, Morocco. Chickpea and lentil samples were collected from Ethiopian Institute of agricultural Research experimental locations. Data of each crop was randomly divided into two sets, a training set (75% of the data) and a deployment set (25% of the data). Crude protein, NDF, ADF and ADL were regressed on ME and Box-cox transformed ME of the training sets to generate prediction models. Coefficients of these models were used to calculate residuals and prediction error (PE) in both training and deployment sets. Criteria used in the screening algorithm were low PE (95th percentile of PE≤4) and homogenous residuals in both training and deployment sets.

Barley and chickpea models were unable to predict ME of deployment samples with a 95th percentile of PE less than 4. Heterogeneity of residuals of the deployment set was found in lentil model (positive residuals= 64% of overall residuals). Accordingly, chemical composition from NIR is a poor predictor for ME of straws of barley, chickpea and lentil to formulate rations for farm management and a direct measurement of ME of these straws is still required.

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Can Ruminant Metabolizable Energy of Barley, Chickpea and Lentil Straw be Predicted Using Chemical Composition?

1 Introduction

In the predominantly mixed crop-livestock systems of developing countries, straw is key feed for livestock in terms of quantity and quality especially during the dry season. Grain yield of one ton is associated with approximately 1.33 t of straw in barley (Sundstøl, 1988), 1.75-8.74 t of straw in chickpea (Wamatu et al., 2017a) and 1.68 - 9.33 t of straw in lentil (Wamatu et al., 2017b). In dry areas of the West Asia and North Africa region, straw constitutes a considerable proportion of diets of sheep in summer and winter (ICARDA, 1986).

Energy content of feeds is important to determine the optimal level of incorporation of the feeds into diets of ruminants. Energy content is also valuable information for pricing straw for marketing purposes. Farmers in India price sorghum stover according to actual or estimated fodder quality (Blümmel & Rao, 2006). Blümmel & Rao (2006) reported that digestibility of stover, which is closely related to energy content, explained 75% of variation in stover prices. Usable energy content of forages available for ruminants is expressed as metabolizable energy (ME) (CSIRO, 2007). Metabolizable energy of a given feed is traditionally determined by subtracting energy of feces, urine and methane from gross energy (Kearl, 1982) but is now commonly assessed in vitro via the Hohenheim Gas Production method (Menke & Steingass, 1988). Some feeding standards use ME to express energy content of feeds like Kearl, (1982) and CSIRO, (2007) while other standards use it to estimate net energy (NRC, 2007).

Gas production technique is an accurate method to determine ME of feeds which based on recording gas emission from an incubation of 0.2g of sample in 100 ml of rumen fluids for 24 h (Menke & Steingass, 1988). Measurement of gas production method requires specialized apparatus, access to rumen fluid, technical skill and is time consuming, requiring a minimum of 24 hours.

Wide varietal and environmental variation in chemical composition and energy content of straw was reported in barley (Capper, 1988), chickpea (Wamatu et al., 2017a) and lentil (Wamatu et al., 2017b). Table 1 presents the chemical composition of barley, chickpea and lentil straw which gives a chance to predict ME using a simple model.

Early attempt to predict ME of feed for poultry nutrition is traced to 1956 (Carpenter & Clegg, 1956). Anderson et al. (2012) have reported that ME of corn coproducts for pigs could be predicted using chemical composition. Similarly, Armstrong et al. (1964) tried to predict ME of dried grasses for sheep fattening using apparent digestibility and chemical compositions. Metabolizable energy content for ruminant of sugar cane, sugar cane silage, soybean silage, mombaça silage (*Panicum maximum* cv. Mombaça), corn silage, Tifton-85 hay (Cynodon spp.) and chopped elephant grass (*Pennisetum purpureum* cv. Cameroon) was predicted using chemical composition (Magalhães et al., 2010). However, these models cannot be used to predict ME for other feeds and individual prediction equations of ME for ruminants must be identified for feed other stuffs (Robinson et al., 2003).

According to our knowledge, there are no studies identified the potential of chemical composition of barley, chickpea and lentil straw to predict ME. Therefore, this study aims to determine robust and accurate models to predict ME of barley, chickpea and lentil straw using chemical composition.

2 Materials & Methods

2.1 Sampling and chemical analysis of straw

Samples of barley straw representing 1933 genotypes (one sample per genotype) were collected from field experiments in Marchouch (33°33’38.2”N 6°41’0” 24.7”W), and Jemma-Shaim (32°21’9.3”N 8°50’32”W) research stations in Morocco during the 2016-2017 season genotypes included 1017 two-row genotypes, 912 six-row and 4 three-row genotypes. A total of 487 (79 genotypes) chickpea and 489 lentil (66 genotypes) samples were collected from 7 and 8 multi-locational trials respectively in Akaki (08°53’N 38°49’E; 2200 m.a.s.l.), Debre Zeit (08°44’N 38°58’E; 1900 m.a.s.l), Chefe Donsa (08°57’N 39°06’E; 2450 m.a.s.l) and Minjir (08°44’N 38°58’E; 1810 m.a.s.l), Ethiopia. Samples were ground to pass through a 1 mm screen and scanned using near infrared reflectance spectroscopy (FOSS 5000 with WINISI II software) to measure crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and ME using equations calibrated and validated for a wide range of barley, chickpea and lentil straws. The performance of the near infrared reflectance spectroscopy prediction equations is presented in Table 1. For equations’ calibration, CP was analyzed according to AOAC (2005) (method 954.01 using Kjeldahl (protein/nitrogen) Model 1026, Foss Technology Corp) NDF was assayed without a heat stable amylase and expressed inclusive of residual ash (Van Soest et al., 1991), ADF was analyzed according to Van Soest et al. (1991)
and expressed exclusive of residual ash, ADL was determined by solubilization of cellulose with sulphuric acid according to Van Soest et al. (1991) and ME were measured in rumen microbial inoculum using the in vitro gas production technique as described by Menke & Steingass (1988). All samples were analyzed at the International Livestock Research Institute laboratory in Addis Ababa, Ethiopia. Details on the near infrared reflectance spectroscopy equations used in this study are presented in Table 1.

### Table 1 Performance of near infrared reflectance spectroscopy prediction models

<table>
<thead>
<tr>
<th>Crop</th>
<th>Standard error of calibration (%)</th>
<th>Standard error of prediction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chickpea straw (n=190)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>0.21</td>
<td>0.425</td>
</tr>
<tr>
<td>NDF</td>
<td>0.85</td>
<td>1.3</td>
</tr>
<tr>
<td>ADF</td>
<td>0.64</td>
<td>1.09</td>
</tr>
<tr>
<td>ADL</td>
<td>0.22</td>
<td>0.36</td>
</tr>
<tr>
<td>ME</td>
<td>0.06</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>Lentil straw (n=111)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>0.6</td>
<td>0.62</td>
</tr>
<tr>
<td>NDF</td>
<td>2.13</td>
<td>2.2</td>
</tr>
<tr>
<td>ADF</td>
<td>1.88</td>
<td>1.83</td>
</tr>
<tr>
<td>ADL</td>
<td>0.59</td>
<td>0.63</td>
</tr>
<tr>
<td>ME</td>
<td>0.996</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Barley straw (n=105)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>0.37</td>
<td>0.508</td>
</tr>
<tr>
<td>NDF</td>
<td>2.26</td>
<td>2.38</td>
</tr>
<tr>
<td>ADF</td>
<td>1.83</td>
<td>2.26</td>
</tr>
<tr>
<td>ADL</td>
<td>0.47</td>
<td>0.68</td>
</tr>
<tr>
<td>ME</td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber.

### 2.2 Statistical analyses

Data of every crop was divided into two different sets, a calibration set (~85% of the data) and a validation set (~15% of the data) using Puchwein (1988) algorithm. The calibration set was used to develop prediction models and the validation set was used to determine the accuracy of the models in predicting ME of new set of samples.

Interquartile range method (Zwillinger & Kokoska, 2003) was used to identify the existence of outliers using the following equation:

Lower bound = Q1 - (IR×1.5)

Upper bound = Q3 + (IR×1.5)

Where Q1 and Q3 are the first and the third quartiles respectively and IR is the interquartile range. Observations of LW which fall out these boundaries were considered outliers.

The probability distribution of ME in the training data set was depicted using the normal Q-Q plot. Box-cox procedure was used to confirm whether a power transformation of ME in the training set would increase predictability of constructed models (Box & Cox, 1964). The optimum power of transformation of ME was identified using a likelihood maximized Box-cox transformation with boundaries of -3 and +3 and a step of 0.25 (Box & Cox, 1964). Crude protein, NDF, ADF and ADL were used to construct linear models to predict ME in each crop. Coefficients of each constructed models were used to calculate residuals. The prediction error (PE) of each model was calculated using calibration set as follows:

\[
PE = 100 \times \left( \frac{ME_p - ME_m}{ME_m} \right)
\]

Where ME\(_p\) and ME\(_m\) are predicted and measured ME respectively.

Similarly, the validation error (VE) of the models was calculated using the validation set. The prediction models were screened in a stepwise approach which included residuals’ magnitude (PE and VE ≤4) and homogeneity (independence of PE and VE from ME (r<0.66) and the symmetric distribution of residuals around zero). All statistical analyses were carried out using the Statistical Analysis System (SAS, 2003).

### 3 Results

All observations in the data had ME (MJ/kg) which lays within the outliers’ boundaries which were 5.9-9.2 for barley, 5.8-8.9 for chickpea and 6.51-10.1 for lentil (Table 2a, 2b). Figure 1 shows the normal Q-Q plot of ME in barley, chickpea and lentil. Normal Q-Q plot of ME shows that distribution of ME was close to normal with some skewness in barley, chickpea and lentil. Results of Box-cox transformation procedure are presented in Table 3. Lambda which had the highest log-likelihood value was different form 1 in models of all crops. Relation between chemical composition and ME are presented in Table 4a,b and Figure 2. The 95th percentile of PE of models with non-transformed ME in all crops was higher than 4 (Table 5a). When ME was
Can Ruminant Metabolizable Energy of Barley, Chickpea and Lentil Straw be Predicted Using Chemical Composition?

Table 2a Chemical composition and metabolizable energy content of barley, chickpea and lentil straw samples of the training set

<table>
<thead>
<tr>
<th>Crop</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>69.2</td>
<td>50.3</td>
<td>98.1</td>
<td>6.24</td>
</tr>
<tr>
<td>NDF (g/kg DM)</td>
<td>738</td>
<td>684</td>
<td>781</td>
<td>12.7</td>
</tr>
<tr>
<td>ADF (g/kg DM)</td>
<td>485</td>
<td>429</td>
<td>532</td>
<td>14.1</td>
</tr>
<tr>
<td>ADL (g/kg DM)</td>
<td>71.4</td>
<td>49.7</td>
<td>90.3</td>
<td>5.74</td>
</tr>
<tr>
<td>ME (MJ/kg DM)</td>
<td>6.98</td>
<td>6.06</td>
<td>8.09</td>
<td>0.275</td>
</tr>
<tr>
<td>Chickpea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>72.5</td>
<td>29.4</td>
<td>216</td>
<td>34.2</td>
</tr>
<tr>
<td>NDF (g/kg DM)</td>
<td>699</td>
<td>478</td>
<td>798</td>
<td>58.4</td>
</tr>
<tr>
<td>ADF (g/kg DM)</td>
<td>457</td>
<td>210</td>
<td>557</td>
<td>62.9</td>
</tr>
<tr>
<td>ADL (g/kg DM)</td>
<td>118</td>
<td>55.7</td>
<td>164</td>
<td>17.5</td>
</tr>
<tr>
<td>ME (MJ/kg DM)</td>
<td>7.37</td>
<td>6.16</td>
<td>9.56</td>
<td>0.511</td>
</tr>
<tr>
<td>Lentil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>496</td>
<td>369</td>
<td>644</td>
<td>48.3</td>
</tr>
<tr>
<td>NDF (g/kg DM)</td>
<td>358</td>
<td>272</td>
<td>506</td>
<td>42.6</td>
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<tr>
<td>ADF (g/kg DM)</td>
<td>88.5</td>
<td>63.2</td>
<td>142</td>
<td>15.4</td>
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<tr>
<td>ADL (g/kg DM)</td>
<td>86.9</td>
<td>34.7</td>
<td>156</td>
<td>25.9</td>
</tr>
<tr>
<td>ME (MJ/kg DM)</td>
<td>8.35</td>
<td>7</td>
<td>9.5</td>
<td>0.45</td>
</tr>
</tbody>
</table>

SD: standard deviation; ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber.

Table 2b Chemical composition and metabolizable energy content of barley, chickpea and lentil straw samples of the deployment set

<table>
<thead>
<tr>
<th>Crop</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>26</td>
<td>14.3</td>
<td>46.6</td>
<td>4.7</td>
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<tr>
<td>NDF (g/kg DM)</td>
<td>795</td>
<td>721</td>
<td>843</td>
<td>19.7</td>
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<tr>
<td>ADF (g/kg DM)</td>
<td>553</td>
<td>470</td>
<td>601</td>
<td>21.8</td>
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<tr>
<td>ADL (g/kg DM)</td>
<td>87.2</td>
<td>54.1</td>
<td>102</td>
<td>6.57</td>
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<tr>
<td>ME (MJ/kg DM)</td>
<td>6.09</td>
<td>5.19</td>
<td>7.29</td>
<td>0.331</td>
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<tr>
<td>Chickpea</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>72.5</td>
<td>29.4</td>
<td>216</td>
<td>34.2</td>
</tr>
<tr>
<td>NDF (g/kg DM)</td>
<td>699</td>
<td>478</td>
<td>798</td>
<td>58.3</td>
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<td>55.7</td>
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<tr>
<td>ME (MJ/kg DM)</td>
<td>7.36</td>
<td>6.15</td>
<td>9.56</td>
<td>0.511</td>
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<tr>
<td>Lentil</td>
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</tr>
<tr>
<td>CP (g/kg DM)</td>
<td>74.3</td>
<td>45.5</td>
<td>115</td>
<td>16.3</td>
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<tr>
<td>NDF (g/kg DM)</td>
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<td>ADF (g/kg DM)</td>
<td>335</td>
<td>257</td>
<td>456</td>
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<tr>
<td>ADL (g/kg DM)</td>
<td>80.6</td>
<td>66</td>
<td>120</td>
<td>10</td>
</tr>
<tr>
<td>ME (MJ/kg DM)</td>
<td>8.5</td>
<td>6.98</td>
<td>9.86</td>
<td>0.548</td>
</tr>
</tbody>
</table>

SD: standard deviation; ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber.

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Figure 1 Q-Q normal plot of metabolizable energy of barley, chickpea and lentil straw of the training set
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Table 3 Lambda values and their corresponding coefficient of determination and log likelihood values resulted from Box-cox transformation procedure

<table>
<thead>
<tr>
<th>Models</th>
<th>Lambda</th>
<th>Log-likelihood</th>
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</thead>
<tbody>
<tr>
<td>Barley</td>
<td></td>
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</tr>
<tr>
<td>NDF</td>
<td>-0.75</td>
<td>2667</td>
</tr>
<tr>
<td>ADF</td>
<td>-0.5</td>
<td>3097</td>
</tr>
<tr>
<td>ADL</td>
<td>-1</td>
<td>2746</td>
</tr>
<tr>
<td>CP</td>
<td>-1</td>
<td>2311</td>
</tr>
<tr>
<td>Chickpea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>0</td>
<td>1212</td>
</tr>
<tr>
<td>ADF</td>
<td>0.5</td>
<td>1214</td>
</tr>
<tr>
<td>ADL</td>
<td>-1.5</td>
<td>1296</td>
</tr>
<tr>
<td>CP</td>
<td>-0.5</td>
<td>704</td>
</tr>
<tr>
<td>Lentil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>1.25</td>
<td>629</td>
</tr>
<tr>
<td>ADF</td>
<td>0.5</td>
<td>630</td>
</tr>
<tr>
<td>ADL</td>
<td>0.75</td>
<td>48</td>
</tr>
<tr>
<td>CP</td>
<td>2.25</td>
<td>364</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber; R²: coefficient of determination; 0 denotes to Log_{10} transformation.

Table 4a Regression of chemical composition on metabolizable energy of barley, chickpea and lentil straw

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Predictors</th>
<th>Coefficients (standard error)</th>
<th>R²</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>NDF</td>
<td>18.4(0.278)</td>
<td>0.513</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>14.9(0.126)</td>
<td>0.714</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>9.55(0.057)</td>
<td>0.556</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>5.48(0.067)</td>
<td>0.24</td>
<td>3.44</td>
</tr>
<tr>
<td>Chickpea</td>
<td>NDF</td>
<td>12.7(0.099)</td>
<td>0.781</td>
<td>3.24</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>10.65(0.06)</td>
<td>0.785</td>
<td>3.22</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>10.46(0.052)</td>
<td>0.811</td>
<td>3.02</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>6.83(0.036)</td>
<td>0.2352</td>
<td>6.07</td>
</tr>
<tr>
<td>Lentil</td>
<td>NDF</td>
<td>12.2(0.119)</td>
<td>0.708</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>11.5(0.098)</td>
<td>0.709</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>10.1(0.094)</td>
<td>0.434</td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>8.07(0.074)</td>
<td>0.035</td>
<td>5.3</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber; R²: Coefficient of determination; CV: coefficient of variation; *: standard error is less than 0.00001; P<0.001 for in all models.
### Table 4b Regression of chemical composition on metabolizable energy of barley, chickpea and lentil straw

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Predictors</th>
<th>Coefficients (SE)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Constant</td>
<td>b</td>
<td>$R^2$</td>
<td>CV%</td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>-0.051(0.007)</td>
<td>0.00038*</td>
<td>0.513</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.163(0.003)</td>
<td>0.00004*</td>
<td>0.713</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.091(0.001)</td>
<td>0.00007*</td>
<td>0.558</td>
<td>2.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17(0.001)</td>
<td>-0.0004*</td>
<td>0.242</td>
<td>3.42</td>
<td></td>
</tr>
<tr>
<td>Chickpea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>1.17(0.006)</td>
<td>-0.00043*</td>
<td>0.769</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.305(0.011)</td>
<td>-0.0013*</td>
<td>0.777</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.381(0.001)</td>
<td>-0.000162*</td>
<td>0.2060</td>
<td>2.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.38(0.001)</td>
<td>-0.00016*</td>
<td>0.2060</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td>Lentil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>22.4(0.253)</td>
<td>-0.017*</td>
<td>0.71</td>
<td>3.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.44(0.017)</td>
<td>-0.002*</td>
<td>0.712</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.66(0.041)</td>
<td>-0.009*</td>
<td>0.436</td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110(2.33)</td>
<td>0.105(0.026)</td>
<td>0.035</td>
<td>11.8</td>
<td></td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy transformed according to results of Box-Cox procedure; NDF: neutral detergent fiber; CV: coefficient of variation; *: standard error is less than 0.00001; P<0.001 for in all models.

Figure 2 Metabolizable energy as a function of chemical composition of barley, chickpea and lentil straw.

ADF: acid detergent fiber; DM: dry matter; ME: metabolizable energy; NDF: neutral detergent fiber
Table 5a Prediction error of models constructed to predict metabolizable energy of barley, chickpea and lentil straw using chemical composition

<table>
<thead>
<tr>
<th>Crop</th>
<th>Predictor</th>
<th>75th</th>
<th>90th</th>
<th>95th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>NDF</td>
<td>3.2</td>
<td>4.41</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>3.73</td>
<td>4.96</td>
<td>6.02</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>2.94</td>
<td>4.29</td>
<td>5.05</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>3.97</td>
<td>5.79</td>
<td>6.83</td>
</tr>
<tr>
<td>Chickpea</td>
<td>NDF</td>
<td>3.19</td>
<td>4.84</td>
<td>6.23</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>3.21</td>
<td>5.31</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>3.21</td>
<td>4.63</td>
<td>6.04</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>3.19</td>
<td>4.84</td>
<td>6.23</td>
</tr>
<tr>
<td>Lentil</td>
<td>NDF</td>
<td>3.25</td>
<td>4.89</td>
<td>5.81</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>3.35</td>
<td>4.99</td>
<td>5.66</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>5.08</td>
<td>6.56</td>
<td>7.45</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>6.04</td>
<td>8.7</td>
<td>10.6</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy; NDF: neutral detergent fiber.

Table 5b Prediction and validation errors of models constructed to predict metabolizable energy of barley, chickpea and lentil straw using chemical composition

<table>
<thead>
<tr>
<th>Crop</th>
<th>Predictor</th>
<th>PE</th>
<th>VE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>75th</td>
<td>90th</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75th</td>
<td>90th</td>
</tr>
<tr>
<td>Barley</td>
<td>NDF</td>
<td>2.95</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>1.39</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>2.94</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>3.91</td>
<td>5.76</td>
</tr>
<tr>
<td>Chickpea</td>
<td>NDF</td>
<td>1.57</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>1.62</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>4.75</td>
<td>6.74</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>3.09</td>
<td>4.89</td>
</tr>
<tr>
<td>Lentil</td>
<td>NDF</td>
<td>4.07</td>
<td>6.19</td>
</tr>
<tr>
<td></td>
<td>ADF</td>
<td>0.16</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>ADL</td>
<td>3.78</td>
<td>4.91</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>13.6</td>
<td>20.2</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; ADL: acid detergent lignin; CP: crude protein; ME: metabolizable energy transformed according to results of Box-cox procedure; PE: prediction error; VE: validation error.
Table 6 Residuals’ distribution and correlation between the dependent variable and prediction and validation error of the constructed models in training and deployment sets of barely, chickpea and lentil

<table>
<thead>
<tr>
<th>Crop</th>
<th>Predictor</th>
<th>Correlation with the dependent variable</th>
<th>Negative residuals (%)</th>
<th>Calibration</th>
<th>Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>ADF</td>
<td>-0.268*</td>
<td>71.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickpea</td>
<td>NDF</td>
<td>-0.1*</td>
<td>47.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickpea</td>
<td>ADF</td>
<td>-0.1*</td>
<td>44.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentil</td>
<td>ADF</td>
<td>-0.054</td>
<td>-0.107*</td>
<td>50.9</td>
<td>36</td>
</tr>
</tbody>
</table>

ADF: acid detergent fiber; NDF: neutral detergent fiber; *: P≤0.05.

Figure 3 Relationship between the dependent variable and prediction error of constructed models of barley, chickpea and lentil training straw samples.

ADF: acid detergent fiber; DM: dry matter; ME: metabolizable energy; NDF: neutral detergent fiber; PE: prediction error; TADF: ADF model with transformed dependent variable; TNDF: neutral detergent fiber model with transformed dependent variable

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transformed, the models with a 95th percentile of PE less than 4 were ADF model (TADF) in barley, chickpea and lentil. The NDF model with transformed ME (TNDF) predicted ME of chickpea straw with a 95th percentile of PE less than 4 (Table 5b).

Correlation between PE and the dependent variable in TADF model in barley, TNDF in chickpea, TADF in chickpea and TADF in lentil was weak (r<0.27; P<0.05) in all crops (Table 6). Distribution of residuals of selected models around zero is presented in Table 6. Frequencies of positive and negative calibration residuals were similar in TNDF model in chickpea, TADF model in chickpea and TADF model in lentil. In barley, negative calibration residuals were dominant in TADF model (71.8%).

An examination of figure 3 and figure 4 shows that PE of TADF model in barley, TNDF model in chickpea, TADF model in chickpea and TADF model in lentil did not agglomerate around specific values of the dependent variable.

The 95th percentile of VE was higher than 4 in TADF model in barley, TNDF and TADF models in chickpea but less than 4 in TADF model in lentil (Table 5b). The Correlation between the dependent variable and VE was weak in TADF model in lentil (r=-0.107). Positive validation residuals dominated negative validation residuals in TADF model in lentil (64%) (Table 6). Figure 4 shows that there were no drifts in VE of TADF in lentil nor systematic relationship between VE and the dependent variable.

4 Discussion and conclusions

Distribution of ME of all crops was deviated from normal as shown in Q-Q plots. This result is confirmed by results of Box-cox transformation procedure which showed that a power transformation of ME might increase the accuracy of prediction of ME using chemical composition. This agrees with McDonald (2009), Lesosky et al. (2013) and Goopy et al. (2017) who reported that transforming the response variable improved accuracy of simple linear regression model in predicting live weight of cattle using heart girth. Accordingly, non-transformed and transformed ME were regressed on chemical composition parameters to construct prediction models.

Metabolizable energy of commercially available forages ranges from 10 to 12.5 MJ/kg (Warren, 2018 personal communication – Unpublished data). Therefore, a difference of 0.5 MJ/kg ME would have a great impact on the resultant ration as 55 - 60% of the dry matter of the diet will be comprised of forages in dairy livestock (Warren, 2018 personal communication - Unpublished data). Accordingly, a maximum of 4% error on a dry matter basis for ration formulation for purposes of farm management, is accepted when ME is estimated (Warren, 2018 personal communication - Unpublished data).

All models with non-transformed response variable could not be used to predict ME to formulate rations for research and farm management as their 95th percentile of PE were higher than 4%. However TADF model in barley predicted ME of 95% of
prediction set samples with a PE less than 4 and PE was independent of ME, distribution of residuals around 0 was asymmetric with a dominance of negative residuals. That means ME of barely straw (~72%) tends to be underestimated by TADF model.

The prediction error of TNDF and TADF models in chickpea were less than 4 and residuals were homogenous. However, both TNDF and TADF models predict ME of 95% of chickpea validation samples with VE higher than 4. In lentil, the 95th percentile of the PE and VE in TADF model was less than 3 and the residuals were homogenous, however, positive residuals dominated validation samples (64%). That means TADF model overestimated almost two third of lentil straw samples in the validation set. Accordingly, NDF, ADF, ADL and CP are poor predictors for straw ME in barley, chickpea and lentil and direct estimation of ME of these straws is still required.

Relationship between chemical composition and digestibility of straw is expected to be affected by morphological structure. Precise prediction of ME of straw might be achieved using morphology-based equations. On that account, prediction equations of ME of morphological fractions of barley, chickpea and lentil straw using chemical composition has to be studied.

Conflict of Interest
Authors declare no conflict of interest regarding publication of this paper.

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ASTHENOZOOSPERMIC BUFFALO (Bubalus bubalis) SPERM TAIL IS ASSOCIATED WITH LOWER EXPRESSION OF TEKTIN-2 AND SPAG6 AND HIGHER EXPRESSION OF SPECIFIC TYROSINE PHOSPHORYLATED PROTEINS

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ABSTRACT

Study of motility-associated proteins is a direct approach to decode the underlying mechanisms of the motility-regulation pathway in a mammalian spermatozoon. In this aspect, attempts were made to characterize and study the expression pattern of two candidate motility-associated proteins of mammalian sperm such as Tektin-2 and SPAG6 and post-translationally modified tyrosine phosphorylated proteins between the two defined groups of buffaloes: normozoospermic (NS) and asthenozoospermic (AS). 1-D Western blot demonstrated three Tektin-2 immunoreactive bands (95.3, 55.7 and 33.0 kDa) in buffalo sperm tails. Of these, the intensity of 95.3 kDa band reduced significantly (P ≤ 0.05) in sperm tail of AS group as compared to that of NS group. Similarly, the sperm tail demonstrated a single SPAG6-immunoreactive band of 31.6 kDa that reduced significantly (P ≤ 0.05) in sperm tail of AS group as compared to that of NS group. Both AS and NS group sperm tail demonstrated at least five tyrosine phosphorylated proteins (89.5, 53.3, 44.5, 30.9 and 16.7 kDa). Of these, the intensity of 44.5 kDa band was significantly higher (P ≤ 0.05) in AS group as compared to that of NS group. Interestingly, within the AS group, degree of expression of 53.3 kDa tyrosine phosphorylated protein was bull-specific. The findings of the present study have demonstrated that AS group of buffalo bulls are associated with lower expression of Tektin-2 and SPAG6 and higher expression of specific tyrosine phosphorylated proteins.

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KEYWORDS
Asthenozoospermia
Buffalo
Motility
SPAG6
Sperm
Tektin-2
Tyrosine phosphorylated proteins

* Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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1 Introduction

Fertilization in mammals is achieved by the fusion of a motile sperm and a reproductively mature egg through a highly orchestrated but less understood process. In cattle, about half of the fertilization failure is attributed to defective bull (Park et al., 2012). Moreover in a herd, a substantial number of buffalo bulls suffer from low sperm motility called asthenozoospermia (Guruprasad et al., 2011). However, the precise reason and mechanism for this reduced motility of buffalo sperm are not known. Mammalian spermatozoa, being a terminally differentiated cell can neither undergo transcription nor translation (Toshimori & Eddy, 2015). The principal mechanism by which a mammalian spermatozoon acquires motility is primarily through the post-translational modifications (PTM) of its proteins and metabolic activities (Naresh & Atreja, 2015). Phosphorylation of sperm proteins at tyrosine residues is one of the significant PTM. In human sperm, the tyrosine phosphorylation of most proteins was concentrated to the sperm tail (Ficarro et al., 2003). The flagellar/tail proteins involved in the motility regulation pathway are tyrosine phosphorylated either through cAMP/PKA pathway or calcium signalling pathway (Turner, 2006). Thus, it is of prime interest to study such flagellar/tail proteins of buffalo sperm that are involved in sperm motility regulation and the proteins that are phosphorylated at tyrosine residues.

Tektins are a group of α-helical structural proteins involved in the construction of sperm cilia and flagella and possess a nonapeptide consensus sequence RPNVELCRD conserved right from the sea urchins to humans, suggesting its significant role in sperm functions (Amos, 2008). Basically, three major tektin polypeptides of molecular masses 47, 51 and 55 kDa were originally isolated from sea urchin (Strongylocentrotus purpuratus) sperm flagellar outer doublet microtubules (Linck & Stephens, 1987). Tektins that have been identified in mammals include Tektin 1-5. Tektin-1, a 50 kDa protein, was found to be expressed in mouse spermatocytes and spermatids and localized to the centrosomes of round spermatid and caudal end of maturing spermatozoa (Larsson et al., 2000). Tektin-2 was found to be crucial for integrity of the dynein inner arm and Tektin-2 null mice are infertile due to loss of sperm motility (Tanaka et al., 2004). Tektin-3 deficient male mice demonstrated reduced progressive sperm motility; however other aspects like sperm count and reproductive status remain undisturbed (Roy et al., 2009). Tektin-4 is essential for the organized beating of sperm flagellum and tektin-4 null mice tend to lose progressive motility (Roy et al., 2007). Tektin-5 was found to be closely associated with the inner side of mitochondrial sheath as revealed by immunogold electron microscopy. Tektin-5 was localised specifically to the middle piece of rat sperm flagellum (Murayama et al., 2008). Thus, Tektins play a crucial role in maintenance of sperm motility and fertility. Men with oligoasthenozoospermia demonstrated reduced expression of Tektin-2 than those with normozoospermia (Bhilawadikar et al., 2013).

Sperm associated antigens (SPAGs) are a group of sperm proteins that elicit auto-antibodies and cause infertility in males (Inaba, 2011). Among the identified SPAGs, SPAG6, 8-9, and 11 are associated with male fertility. SPAG6 knockout mice demonstrate impaired sperm motility and male infertility (Sapiro et al., 2002). SPAG6 deficient mice either survive but demonstrate male sterility or die due to hydrocephalus (Teves et al., 2014). A study reported that, SPAG6 is essential for the normal formation and function of immunological synapse, thus demonstrating the significance of SPAG6 to fertility (Cooley et al, 2016). SPAG8 plays an important role in CREM-ACT (cAMP response element modulator- activator) mediated gene transcription during spermatogenesis (Wu et al., 2010). SPAG9 is a sperm surface protein and have been found to play role in sperm-egg interaction (Jagadish et al., 2005); SPAG11 isoforms were abundant in the rat male reproductive tract and implicated to play a role in immunity (Yenugu et al., 2006).

Protein tyrosine phosphorylation is one of the most significant intracellular signalling events associated with sperm motility and capacitation in several species (Visconti et al., 1995; Galantino-Homer et al., 1997; Roy & Atreja, 2008). A 55 kDa sperm protein, identified as glycogen synthase kinase-3α, was found to be tyrosine phosphorylated during epididymal maturation of bovine sperm (Vijayaraghavan et al., 1997; Vijayaraghavan et al., 2000). Tyrosine phosphorylation of two sperm proteins viz. nuclear protein localization protein 4 homologue (68 kDa) and keratin, type-II cytoskeletal 1 (66 kDa) were increased significantly in AS human sperm (Chan et al., 2009). Tyrosine phosphorylation of glucose regulated protein 78 (GRP78) was found to be reduced significantly in sperm of AS human semen (Parte et al., 2012). A 56 kDa protein was found to be tyrosine phosphorylated in cryopreserved bovine sperm that are associated with premature capacitation (Cormier & Bailey, 2003).

To the best of our knowledge, the specific association of various isoforms of Tektin-2, SPAG6 and protein tyrosine phosphorylation to sperm motility has not been explored so far in any ruminant species including buffaloes. Thus, it was hypothesized that the expression pattern of Tektin-2, SPAG6 and protein tyrosine phosphorylation vary between normozoospermic (NS) and asthenozoospermic (AS) groups of buffalo semen.

2 Materials and Methods

Semen samples were collected twice a week from seven Murrah buffalo (Bubalus bubalis) bulls, between 3-5 years of age, maintained under isomanagerial conditions at Nandini Sperm
Station (NSS), Hessarghatta, Bangalore, Karnataka, India. Animal management and semen collection protocols of the sperm station were as per the relevant guidelines and regulations of the state government.

2.1 Collection of buffalo semen and analysis of sperm motility

Semen ejaculates from Murrah buffalo bulls were collected using an artificial vagina (IMV, France) maintained at 40°C. Shortly after collection, semen samples were assessed for their mass activities by light microscopy at 10 X magnifications. A minor aliquot of semen sample was diluted (1:10) in sperm-Tyrode’s albumin lactate pyruvate hepes buffer (sp-TALPH) media, pH 7.4 [100-mM NaCl, 3.1-mM KCl, 0.4-mM EDTA di-sodium salt, 0.4-mM MgCl2, 6H2O, 0.3-mM NaH2PO4.2H2O, 21.6-mM Sodium lactate, 2-mM CaCl2.2H2O, 1-mM Sodium pyruvate, 40-mM hepes, 10-mM NaHCO3 and 1mg/mL (w/v) polyvinyl alcohol (PVA, 30-70 kDa)] and the progressive motility of sperm was evaluated by computer assisted semen analyser (CASA, version 3.2.0; Microptic, Barcelona, Spain) (Dott & Foster, 1979). Based on the percentage of progressive motility of sperm as assessed by CASA, buffalo semen samples were categorized in to two groups viz., normozoospermic (NS, progressive motility ≥70%, n=8) and asthenozoospermic (AS, progressive motility ≤ 40%, n=10). Each of the samples for NS and AS groups was from different bull. Two ejaculates of the same bull collected at 30 minutes interval were pooled and this constituted one semen sample. Semen samples having progressive motility above 40% and below 70% were excluded for the study.

2.2 Separation of buffalo sperm head and tail

The major aliquot of semen was centrifuged to separate sperm pellet and seminal plasma as described earlier (Divyashree & Roy, 2018). The sperm pellet obtained was subjected to sonication followed by sucrose gradient centrifugation to separate sperm head and tail according to the method of O’Brien & Bellve (1980) as modified by Carrera et al. (1994). Briefly, the sperm pellet was washed once with S-EDTA buffer, pH 6.0 [75 mM NaCl, 1% (w/v) SDS, 24 mM EDTA, pH 6.0] and resuspended in appropriate volume of S-EDTA buffer, pH 6.0. The sperm suspension was then sonicated (VCX130; Sonic Vibra Cell, Sonics & Materials Inc., Newtown, U.S) at 40 % amplitude for 8 cycles at 4°C (15 sec pulse ON, 10 sec pulse OFF). The suspension was then vortexed (Spinix, Tarsons, Kolkata, India) for 1 minute each and stored at 4°C for 1 hour with intermittent vortexing at 10 minutes interval. The suspension was then observed for separation of head and tail under phase contrast microscopy (Eclipse-80i, Nikon Instruments Incorporation, Tokyo, Japan). The suspension was then layered on 5X volumes of 1.6 M sucrose in S-EDTA and the tubes were centrifuged at 5000X g for 1 hour at 20°C. The SDS-resistant tail and having lowest intensity was observed for separation of head and tail according to the method of Divyashree & Roy (2018). The sperm pellet obtained was subjected to sonication with 1mg/mL (w/v) polyvinyl alcohol (PVA, 30-70 kDa) and the proteins of sperm were analysed by SDS-PAGE and Western blotting as modified by Carrera et al. (1994). The proteins separated in SDS-PAGE were transferred on to the Immobilon-P PVDF membrane ( pore size, 0.45 mm) as described earlier (Divyashree & Roy, 2018). The blots were incubated with goat anti-human Tektin-2 and SPAG6 polyclonal antibodies @ 2.6 μg/mL and 2.0 μg/mL, respectively (sc-160104, sc-165528; Santa Cruz Biotech, Dallas, Texas, US); mouse monoclonal anti-phosphotyrosine antibody @ 1.0 μg/mL (P1869, Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at room temperature. The amino acid sequence homologies between human and predicted buffalo Tektin-2 and SPAG6 are 91% and 96% respectively (https://blast.ncbi.nlm.nih.gov/Blast). After washing with TBS-T, the membranes were incubated in secondary antibodies; rabbit anti-goat immunoglobulin conjugated with horseradish peroxidase (dilution 1:1,60,000 and 1:1,80,000 for Tektin2 and SPAG6 respectively; A5420, Sigma-Aldrich, St. Louis, MO, USA) and anti-mouse immunoglobulin conjugated with horseradish peroxidase (dilution 1:50,000; A2554, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature and washed again with TBS-T. Subsequently, the membrane was treated with Clarity Western ECL substrate (Bio-Rad Laboratories Inc, Hercules, CA, USA) to visualize the peroxidase activities, as per manufacturer’s instructions. The enhanced chemiluminescence signal that developed on the membrane was captured on X-ray films (Fujifilm Corporation, Tokyo, Japan) in a dark room. The exposure schedules were separated as an interface in the sucrose gradient which was collected and stored at -20°C for further use.

2.3 SDS-PAGE and Western blot for detection of Tektin-2, SPAG6 and tyrosine phosphorylated proteins

Sperm tail suspension was added with equal volumes of 2x reducing sample of Laemmli (1970) with 100 mM DTT as the reducing agent. The solubilised proteins were resolved by 10% uniform reducing SDS-PAGE according to the method of Laemmli (1970). The molecular masses of separated proteins were determined by applying standard protein molecular weight markers (Precision Plus dual colour standards, Bio-Rad, Hercules, California, US) in a reference well.

Proteins separated by SDS-PAGE gels were transferred on to the Immobilon-P PVDF membrane (pore size, 0.45 mm) as described earlier (Divyashree & Roy, 2018). The blots were incubated with goat anti-human Tektin-2 and SPAG6 polyclonal antibodies @ 2.6 μg/mL and 2.0 μg/mL, respectively (sc-160104, sc-165528; Santa Cruz Biotech, Dallas, Texas, US); mouse monoclonal anti-phosphotyrosine antibody @ 1.0 μg/mL (P1869, Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at room temperature. The amino acid sequence homologies between human and predicted buffalo Tektin-2 and SPAG6 are 91% and 96% respectively (https://blast.ncbi.nlm.nih.gov/Blast). After washing with TBS-T, the membranes were incubated in secondary antibodies; rabbit anti-goat immunoglobulin conjugated with horseradish peroxidase (dilution 1:1,60,000 and 1:1,80,000 for Tektin2 and SPAG6 respectively; A5420, Sigma-Aldrich, St. Louis, MO, USA) and anti-mouse immunoglobulin conjugated with horseradish peroxidase (dilution 1:50,000; A2554, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature and washed again with TBS-T. Subsequently, the membrane was treated with Clarity Western ECL substrate (Bio-Rad Laboratories Inc, Hercules, CA, USA) to visualize the peroxidase activities, as per manufacturer’s instructions. The enhanced chemiluminescence signal that developed on the membrane was captured on X-ray films (Fujifilm Corporation, Tokyo, Japan) in a dark room. The exposed films were processed and developed.

2.4 X-ray film imaging and densitometry of detected proteins

Following chemiluminescence detection of the proteins, the X-ray films were photographed by a gel documentation system (LAS-3000; Fujifilm, Tokyo, Japan). The molecular mass and densitometric analyses of the detected protein bands were performed using the Multi Gauge analysis software (version 2.2, Fujifilm, Tokyo, Japan). The band having lowest intensity was assigned a base value of 100 to determine the relative intensities of other bands with respect to the reference band. Beta-actin and alpha-tubulins are not good loading controls for immunoblotting
for various reasons (Dittmer & Dittmer, 2006; Thacker et al., 2016; Moritz, 2017). Hence, Ponceau S staining (Salinovich & Montelaro, 1986) method was used as a loading control for the western blot.

2.5 Statistical analysis

Data obtained were initially tested by Shapiro-Wilk test for their normal distribution. Then the data were analyzed by independent t-test to determine whether there is a statistically significant difference between the means of two groups (NS vs. AS). Both the analyses were performed using SPSS software, version 18.0 (SPSS Inc., Chicago, IL). Data are presented as mean ± SEM. A difference with value P< 0.05 was considered statistically significant.

3 Results

The progressive motility percentage of semen samples of NS and AS group of Murrah buffalo were 82.0±5.05 (77.1-92.7, n=4) and 32.3±3.90 (24.8-37.8, n=3), respectively. Following sucrose gradient centrifugation of buffalo whole sperm pellet in S-EDTA buffer, SDS-resistant tail structures appeared in the interface, whereas sperm heads settled as pellet. SDS-resistant tails were observed under a phase contrast microscope (Eclipse-80i, Nikon Instruments Incorporation, Tokyo, Japan) as longitudinal tubular structures (Figure 1).

The 1-D western blot analysis of Tektin-2 demonstrated at least three immunoreactive protein bands such as 95.3, 55.7 and 33.0 kDa in both NS and AS buffalo sperm tail fraction with different bulls (Figure 2A). Further, densitometric analyses revealed that the intensity of 95.3 kDa Tektin-2 immunoreactive band of AS group sperm tail was significantly lower (P < 0.05) than that of the NS group (Figure 2B). Contrastingly, the intensity of 55.7 and 33.0 kDa band of AS buffalo sperm tail fraction did not vary significantly with that of NS sperm tail fraction.

Western blot detection of SPAG6 revealed a single immunoreactive band of 31.6 kDa in NS and AS group of sperm tail fraction (Figure 3A) and densitometric analysis of the same band showed a significantly lower expression in AS sperm tail fraction as compared to that of NS group (Figure 3B).

Western blot analysis to detect tyrosine phosphorylated proteins in buffalo sperm tail fraction portrayed at least five immunoreactive bands viz., 89.5, 53.3, 44.5, 30.9 and 16.7 kDa (Figure 4A). Tyrosine phosphorylation of a 44.5 kDa sperm tail protein was significantly higher in AS group when compared with that of NS group (Figure 4B). Interestingly, in one bull of AS group, the tyrosine phosphorylation of a 53.3 kDa protein was significantly higher as compared to that of other bulls of the same group as well as all the bulls of the NS group.

Figure 1 Photomicrograph demonstrating sperm tails separated from buffalo spermatozoa following sucrose gradient centrifugation under phase contrast optics. Scale bar =100 µm.
Figure 2A A representative Western blot for detection of Tektin-2 protein in buffalo sperm tail fractions. Sperm tail proteins were separated using uniform 10% SDS-PAGE. Tektin-2 protein was detected using goat anti-human Tektin-2 polyclonal antibody. Approximately, 30 μg of proteins were loaded in each well. MWM: molecular weight markers; NS: normozoospermic sperm tail proteins; AS: asthenozoospermic sperm tail proteins; 2B. Densitometric analysis of 95.3 kDa (NS, n=2; AS, n=3) Tektin-2 protein band as shown in Fig.2A. The data are expressed as the mean ± standard error of the mean. Different letters above bars indicate significant differences (P<0.05) between different groups.

Figure 3A A representative Western blot for detection of SPAG6 protein in buffalo sperm tail fractions. Sperm tail proteins were separated using uniform 10% SDS-PAGE. SPAG6 protein was detected using goat anti-human SPAG6 polyclonal antibody. Approximately, 30 μg of proteins were loaded in each well. MWM: molecular weight markers; NS: normozoospermic sperm tail proteins; AS: asthenozoospermic sperm tail proteins. 3B. Densitometric analysis of 31.6 kDa (NS, n=4; AS, n=3) SPAG6 protein band of Fig.3A. The data are expressed as the mean ± standard error of the mean. Different letters above bars indicate significant differences (P<0.05) between different groups.
Discussion and conclusions

The present study evidenced differential expression of Tektin-2, SPAG6 and tyrosine phosphorylated proteins in two different groups of buffalo sperm tails. The AS group of buffalo semen was associated with significantly lower expression of Tektin-2 and SPAG6 and higher expression of two specific tyrosine phosphorylated proteins (44.5 and 53.3 kDa). In the present study, both groups of buffalo bull sperm demonstrated three immunoreactive Tektin-2 isoforms (95.3, 55.7, and 33 kDa). Earlier, the molecular mass of Tektin-2, so far reported in Homo sapiens, was 46 and 56 kDa (Iguchi et al., 2002). This variation in the isoforms may be attributed to species difference or cross reactivity of the heterologous anti-human Tektin-2 polyclonal antibody used in the study. Among the three immunoreactive isoforms of Tektin-2 detected by 1-D Western blot, only the 95.3 kDa band demonstrated a significantly lower expression in AS group sperm tails as compared to that of NS sperm tails. The antibody might have also cross-reacted with intermediate filament networks/ intermediate filament proteins as suggested earlier (Chang & Piperno., 1987; Steffen & Linck., 1989). In a recent study, a 49 kDa Tektin-2 expression was reported to be positively correlated with sperm motility in buffalo bulls (Xiong et al., 2018). However, in present study, at least three molecular weight forms of Tektin-2 immunoreactive protein bands were detected and of these, only the 95.3 kDa band was highly expressed in NS group bulls. Significantly lower expression of a 95.3 kDa Tektin-2 isoforms in AS group sperm tail consolidates the functional significance Tektin-2 in buffalo bulls.

In buffalo, the 31.6 kDa SPAG6 immunoreactive band was significantly reduced in AS group buffalo sperm tails as compared to that of NS group. SPAG6 is an important structural protein of sperm tail as SPAG6 knockout mice demonstrated impaired sperm motility and male infertility (Sapiro et al., 2002). SPAG6 deficient mice either survive but demonstrate male sterility or die due to hydrocephalus (Teves et al., 2014). Impaired sperm motility of SPAG6 knock-out mice was attributed to the dysfunction of central apparatus and not due to structural abnormalities in the central apparatus.
flagella (Sapiro et al., 2002). Lower expression of SPAG-6 in AS group of buffalo semen may be attributed for lower sperm motility and this specific association of SPAG6 to sperm motility in buffalo has not been reported so far.

To the best of our knowledge, this is the first report to demonstrate the higher expression of tyrosine phosphorylated proteins (53.3 and 44.5 kDa) in AS group buffalo sperm tail as compared to that of the NS group. Apart from higher expression of 53.3kDa band in AS group, there were also differences in the intensities/ expression pattern among the AS group of bulls indicating individual bull-to-bull variation even within a same group. Further, the 44.5 kDa band also displayed significantly higher expression in AS group of buffalo sperm tail as compared to that of NS group. During the course of sperm motility regulation pathway, tyrosine phosphorylation is a significant event that leads the sperm to a motile state (Tash & Bracho, 1998). Significantly higher expression of specific tyrosine phosphorylated proteins in AS group sperm tail may be an indication of early or premature capacitation of sperm, as significantly higher percentage of acrosomes are lost from AS group spermatozoa as compared to that of NS group sperm (unpublished observation). Buffalo sperm has been reported to undergo spontaneous capacitation even in the absence of heparin (Roy & Atreja, 2009). In an earlier study, tyrosine phosphorylation served as an indicator of sperm capacitation status in fresh and cryopreserved stallion and bovine spermatozoa (Pommer & Meyers, 2002; Cormier & Bailey, 2003). Capacitation, being a time-dependent event can affect the stages of fertilization process, if there are any advances or delay in its initiation. Thus, characterization of these highly expressed tyrosine phosphorylated proteins in AS group of buffalo semen is of greater significance. Further, it would be interesting to identify these proteins after 2-Dimensional gel electrophoresis followed by Mass spectrometric identification so as to determine the specific functional significance of tyrosine phosphorylation in the two defined groups of buffalo bulls.

In the present study, attempts were made for the first time to determine the association of buffalo sperm motility with the expression pattern of two structural proteins i.e. Tektin-2 and SPAG6 and specific tyrosine phosphorylated proteins. Higher expression of specific tyrosine phosphorylated proteins in sperm tail of AS group semen may be an indicator of premature capacitation of sperm. Sperm motility being a key requisite for fertility, molecular identification of such motility-associated proteins is a crucial step ahead to unlock the mechanism of reduced motility in AS group of buffalo sperm.

Conflicts of interest

The authors declare that there is no conflict of interest for the contents of the manuscript.

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IMMUNOLOGICAL INTERACTION BETWEEN THE FISH LOUSE *Lepeophtheirus salmonis* AND THE HOST ATLANTIC SALMON *Salmo salar* L.

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ABSTRACT

The salmon louse, *Lepeophtheirus salmonis*, is a crustacean ectoparasite of salmonids and causes significant economic losses in Atlantic salmon farms. This study investigated the cellular inflammatory response, specifically macrophage migration, to the attachment of salmon louse to the epidermis of Atlantic salmon (*Salmo salar*). During the inflammatory response, macrophage infiltration is common at the site of infection in the host macrophages possessing phagocytic activity. The aim of the study was to investigate the immunological response of macrophages to the parasitic excretory and secretory product (E/S). The E/S of the parasites collected from the explants and *in vitro* cultures of parasites and macrophages isolated from fish blood were used. A macrophage migration and a phagocytosis assay were performed to study the inflammatory response. The inhibition of the macrophage migration response (*p*<0.05) and a significantly lower level of phagocytosis (*p*<0.05) were observed. The results indicate that the anti-inflammatory response of the host is elicited during louse infection.

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1 Introduction

The salmon louse, *Lepeophtheirus salmonis*, is a crustacean ectoparasite of salmonids and causes significant economic losses in salmon farms (Rae, 1979; Pike, 1989). The Atlantic salmon is more susceptible than other salmon species to *Lepeophtheirus* infections (Johnson & Albright, 1992a; Johnson & Albright, 1992b). Coho salmons treated with corticosteroids are susceptible to infection by sea lice due to a reduced inflammatory response in the fins and gills (Johnson & Albright, 1992a; Johnson & Albright, 1992b). Parasites have a complex lifecycle and have different defense strategies to successfully survive in the host via immunosuppressive tactics (Ko, 1989). Cell culture is generally considered to be a useful technique for biochemical (Kusuda et al., 1991) and biotechnological studies related to invertebrate pathology (Maramorosch, 1987), pharmacology, and immunology (Taylor & Baker, 1978). The development of *in vitro* cell cultures was established using tissues from shrimp (Luedeman & Lightner, 1992), lepidopteran, and dipteran insect cultures (Lee & Hou, 1992), as well as tick cultures (Munderloh et al., 1994). Most *in vitro* cell culture experiments were performed with the helminth parasite *Schistosoma mansoni* (Bayne et al., 1994). The host signals for the attachment of various ectoparasites and endoparasites to host explants have been studied in the fish parasite copepodid (*Salmincola edwardsii*), as well as cercaria of a fish parasite (*Acanthostomum japonicum*), a duck parasite (*Trichobilharzia ocellata*), and a human parasite (*Schistosoma japonicum*). In previous studies, the primary culture of normal pituitary cells of carp (*Cyprinus carpio*) was found to release gonadotropin during *in vitro* culture (Ribeiro et al., 1983) and the synthesis of *Sarcophaga* lec tin and sarcotoxins in NIH-SAPE-4 was observed in a *Sarcophaga peregrina* embryonic cell line (Komano et al., 1988). The adhesion of parasites on to host surfaces has been widely reported in mammalian cell lines (Kirkpatrick & Svilenov, 1987; Lumb et al., 1988). For endoparasitic protozoans, the attachment of *Cryptosporidium* sporozoites to Madin-Darby canine kidney (MDCK) cells (Lumb et al., 1988) was demonstrated. Furthermore, it has been reported that *Eimeria magna* sporozoites attach to embryonic bovine trachea cells (Jensen & Edger, 1976) and that *Eimeriatenella* develops in Japanese quail embryos (Nakai et al., 1992). *Sarcocystis cruzis* sporozoites have been found to develop in both bovine monocytes and bovine endothelial cells (Speer & Dubey, 1986). Primary cell cultures and immortal cell lines grown in a bovine fetal kidney cell culture (Chang & Gabrielson, 1984) have been established for *Toxoplasma gondii* (Hughes, 1986). The penetration of *Schistosoma mansoni* cercaria into a host material, equivalent to living skin, has been previously demonstrated by Fusco et al. (1986). An inflammatory response and extensive cellular infiltration has been observed during parasitic infection (Paperna & Zwerner, 1982; Johnson & Albright, 1992a). Hosts rejected the parasitic copepods *Lernaea cyprinacea* and *Lernaea polymorpha* (Shariff & Roberts, 1989). In fish, inflammatory responseshave been observed upon injection of inactivated bacteria (Bartos & Sommer, 1981), live bacteria (MacArthur & Fletcher, 1985), extracellular products of *Aeromonas salmonicida* (Ellis et al., 1976), and parasitic diseases (Cawthorn et al., 1990).

Macrophages and neutrophils secrete a variety of products with defensive functions, such as reactive oxygen species (Secombes et al., 1988), cytokines (Clem et al., 1985), leukotriene-1β, tumor necrosis factor-α (Dinarello, 1991), IL-8 (Ribeiro et al., 1983), macrophage derived chemotactic factor (MNCF) (Cuha & Ferreira, 1986), bacterial peptides, and LPS (Cramer et al., 1991). *Glugea* parasitic infection in the three spine stickle back (Weissenberg, 1968) and plaice (McVicar, 1975), and *Cryptobiasalm ositica* infection in rain bow trout (Jones & Woo, 1986) have also been previously investigated. The aim of this study was to understand the immunosuppressive effect of *L. salmonis* cultured *in vitro*, and to determine whether a leucocyte migratory inhibitory factor could be obtained in the supernatant.

2 Materials and Methods

2.1 Experimental Fish

Atlantic salmon (*Salmo salar*) weighing 1 kg and 200 g were obtained from the SOAED marine fish farm and maintained in glass fiber tanks with a constant flow (4 l/min) of continuously aerated sea water at 18°C and fed an appropriate daily diet of BP commercial pelleted food (2% w/w/day). Experimental animals were anaesthetized with benzocaine (Sigma) and euthanized by a sharp blow to the head.

2.2 *In vitro* culture of *L. salmonis*

To allow for copepodid attachment to the *in vitro* host cell line, the culture medium was optimized by determining the copepodid survival. This was measured as activity and cell viability, determined as the spread of confluent cells and the appearance of cells in different media with varying osmolarity. Mean copepodid survival in percentage was calculated by comparing the number of wells showing copepodid survival to total cells containing copepods. The mean survival of cells was calculated based on the number of cells showing confluent cell monolayers exposed to copepodid in the wells. The media used were minimum essential medium (MEM) (Gibco), Leibovitz (L-15) (Gibco), sterile water, phosphate buffered saline (PBS), and insect medium (Sigma) at 10°C. Copepods were sterilized prior to carrying out the *in vitro*
attaching 0.1 (w/v) supernatant at 10°C in a cooled incubator to enable attachment. Observations were carried out at 12 hrs intervals and the attachment was recorded using a camera. The culture medium was replaced with fresh medium every 48 hrs carefully to avoid disturbing the copepods or the cell layer.

### 2.3 Isolation of leucocytes

Leucocytes were isolated from the head kidney (HK), for this, pronephros tissue of the HK was aseptically removed and separated using a 100 µm size nylon mesh (Nitex) with 5 ml 1-15 (Gibco) containing 2% fetal bovine serum (FBS) (Gibco), penicillin/streptomycin (100 units/ml)(Gibco), and 10 units/ml of heparin (Sigma). The cell suspension was placed on a 37-51% Percoll (Pharmacia) discontinuous density gradient and centrifuged at 400 g and 4°C for 35 minutes. The leucocyte cell fraction was separated from the 37-51% Percoll interface was resuspended in L-15 (Gibco) containing 1% FBS (Gibco) and centrifuged at 400 g for 15 minutes. The cell pellet was collected and resuspended in the same medium and the cell number was adjusted to 1 × 10^6 cells/well with L-15 medium [containing 5% FBS and penicillin/streptomycin (100 units/ml)] and dispersed into each well of a 96-well tissue culture plate (Nunc). After 3 hrs incubation at 18°C, non-adherent cells were removed by washing the cell layer with sterile phenol red free HBSS, pH 7.4 (Gibco). The remaining cells were resuspended in L-15 containing 2% FBS with vigorous pipetting.

### 2.4 Production of host chemoattractant containing supernatant

A chemoattractant containing supernatant, as described in the section above, by stimulation with a mixture of 50 µl of 10 µmol calcinophore (Sigma) and 50 µl of 50 µg/ml sea lice culture supernatant (SLSN). Likewise, the series of wells containing leucocyte monolayers were also stimulated with control tissue culture supernatant (TCSN) (80 µg/ml), copepodid crude extract (COPE) (50 µg/ml), or Hank’s balanced salt solution (HBSS), added together with the above mentioned volumes and concentrations of calcinophore. The plate was incubated for 2 hrs at 18°C. The leucocyte supernatant was harvested and centrifuged at 1000 g for 25 min to remove the leucocytes, then the supernatant was aliquoted and stored at -20°C until further use. Atlantic salmon serum and LPS were also used as the host chemoattractant.

### 2.5 Preparation of macrophages treated with parasite products

The macrophages (HK) of Atlantic salmon were isolated as described previously (section 2.3) and washed twice with sterile HBSS. The cell viability was determined using the 0.1 (w/v) trypan blue exclusion method (Phillip, 1973) and the cell number was adjusted to 1 × 10^6 cells/well (1 × 10^3 cell/ml) diluted with HBSS. The cells were then incubated with 100 µl copepodid culture supernatant (SLSN) and 100 µl tissue culture supernatant (80 µg/ml) or copepodid crude protein extract (50 µg/ml) for 2 h at 180°C. Afterwards, the cells were washed thoroughly with sterile HBSS and the viability of cells was observed again as described previously.

### 2.6 Leucocyte migration assay

The migratory responsiveness of freshly isolated Atlantic salmon macrophages (HK) and sea lice culture supernatant-treated macrophages to the above-mentioned host chemoattractant was investigated using a technique initially developed by Boydon (1962). A volume of 200 µl/well chemoattractant was dispensed into triplicate wells of the 24-well chemotaxis chamber (Nucleopore Corp., Cambridge, MA, USA). The chemoattractants were fresh Atlantic salmon serum (1:4 dilution), calcium ionophore stimulated leucocyte supernatant (1:2 dilution), LPS (10 µg/ml), sea lice culture supernatant (SLSN) (80 µg/ml), copepodid crude protein extracts (COPE) (50 µg/ml), and tissue culture supernatant (TCSN) (80 µg/ml). A 3 µm pore size polyvinyl pyrrolidone free polycarbonate filter (Millipore Corp., Bedford, MA, USA) was then placed above the cells. Subsequently, the upper chamber was placed on top of the filter. A volume of 100 µl of 2 × 10^6 cells/well macrophages or macrophages treated with the parasitic supernatants were dispensed into triplicate wells of the upper chamber. The extracted supernatants were added to the lower chamber. The chemotaxis chamber was incubated at 18°C for 3 hrs. This assay was also carried out with untreated macrophages in the upper chamber as well as the sea lice culture supernatant (80 µg/ml) and the copepodid crude protein extract (50 µg/ml) in the respective lower chambers. After the incubation period, the filter was removed and the upper surface was thoroughly washed with HBSS three times to remove any non-adherent cells on the upper surface. The number of the cells attached to the lower surface of the filter showing directional or chemotactic migratory responsiveness measured using a microscope (400x magnification). The data was used to calculate the migratory index.
2.7 Statistical analysis

The chemotaxis assay was performed in triplicate on at least six fish. The data were analyzed using analysis of variance (ANOVA). Mini-tab 10 software was used to analyze the raw data.

3 Results and Discussion

Macrophages and cell viability

The number of macrophages collected from the Percoll gradient did not vary significantly between the fish, with a concentration of $1 \times 10^{10}$ cells/ml/fish. The contaminating cells were red blood cells and thrombocytes. In these cells, less than 5% of neutrophils were found. Macrophage viability was determined immediately after isolation, as well as at 2, 6, 12, and 24 hrs using the trypan blue exclusion method. Both immediately after isolation and 2 hrs later, the cell viability was found to be greater than 80%. After 24 hrs, approximately 70% of the macrophages were viable. After 12 hrs, the cells showed a greatly reduced migratory response compared to that after 6 hrs. The cells isolated after 24 hrs did not migrate whereas the cells collected immediately after isolation showed an extensive migratory response after 2 hrs. This observation must be compared with the observations of Atlantic salmon, coho salmon, and chinook salmon experimentally infected with *L. salmonis*. This demonstrates that Atlantic salmon is more susceptible to *L. salmonis* infection than coho salmon (Johnson & Albright 1992a; Johnson & Albright 1992b). In addition, hydrocortisone-treated coho salmon showed an enhanced susceptibility to *L. salmonis* infection (Johnson & Albright 1992a; Johnson & Albright 1992b). This supports that the copepodid or chalimus might possess an immunosuppressive or anti-inflammatory chemotactic factor, apparently inhibiting cell migration (Figure 4). The chemotactic inhibitory factor may be found in soluble form in the secretory product of sea lice, resulting in a significantly lower migratory responsiveness, when macrophages from sea lice culture supernatant were compared to controls (tissue culture supernatant) in the leucocyte migration assay.

3.2 Treated macrophages and cell viability

The size of the treated macrophages was enlarged compared to untreated macrophages, with a vacuolated cytoplasm, as seen in Figure 1. Some of the macrophages seemed to have a slightly extended cytoplasmic tail. The cell viability was approximately 60-70% and lower than that of untreated cells.

3.3 Leucocyte migration assay

Viable macrophages were treated with SLSN, TCSN, and copepodid extract in order to confirm the effect on macrophage migration towards the chemoattractant. This assay was performed by comparing treated and untreated macrophages in parallel. Untreated macrophages showed a higher migratory responsiveness compared to treated macrophages in all experiments. Untreated macrophages showed a high migratory responsiveness to fresh Atlantic salmon serum used as a chemoattractant, but a low responsiveness to heat-inactivated Atlantic salmon serum (Table 1). The chemotactic responsiveness of treated cells was significantly lower than that of untreated cells in all experiments when serum was used as a chemoattractant ($p<0.05$). However, there was no significant difference in the migration of cells treated with sea lice supernatant (SLSN) or tissue culture supernatant (TCSN) ($p>0.01$).

Table 1 Chemotactic activity (number of macrophages (HK) showing migration) when fresh serum was used as the host chemoattractant at different times following isolation.

<table>
<thead>
<tr>
<th>Incubation period (time in hours)</th>
<th>Chemotactic activity (mean number of cells/ microscopic field)</th>
</tr>
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<tbody>
<tr>
<td>02</td>
<td>63 ± 12</td>
</tr>
<tr>
<td>06</td>
<td>28 ± 05</td>
</tr>
<tr>
<td>12</td>
<td>12 ± 03</td>
</tr>
<tr>
<td>24</td>
<td>00 ± 00</td>
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</tbody>
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Figure 1 Schematic illustration of copepodid (A) Free living stage of copepodid (B) Attached copepodid.
3.4 Ca++ ionophore stimulated leucocyte supernatant

The enhanced migratory responsiveness when the Ca++ ionophore-generated leucocyte supernatant was used as a chemoattractant is shown in Figure 2. In this case, macrophages were stimulated with a Ca++ ionophore as well as lice products. The supernatants generated were harvested and used as potential chemoattractants. However, they did not show any significant difference in responsiveness compared to the use of Ca++ ionophore alone (Figure 2).

The cells treated with copepodid crude protein extract showed a significantly lower responsiveness when LPS was used (p<0.0005) (Figure 3). There was no significant difference in migratory responsiveness between treatment and control groups.

Notably, the migratory responsiveness was significantly lower when only sea lice culture supernatant (SLSN) was used as a chemoattractant rather than the control tissue culture product (TCSN) (p<0.01). The macrophages in the presence of the sea lice culture product also showed significantly lower responsiveness compared to cells in the presence of copepodid crude protein product alone (Figure 4).

3.5 Migratory pattern of macrophages

After the macrophages migrated through the pores of the filter, the macrophages had a characteristic shape. The cells appeared elongated, with projecting cytoplasmic processes and greater plasticity. In most of the cells, the cellular body containing the nucleus preceded a highly elongated cytoplasmic tail. The migratory pattern of the untreated cells was found to differ from that of the treated cells. Untreated cells showed a highly extended cytoplasmic tail compared to treated cells, indicating that the macrophage receptors are blocked by the crude factors in the culture products.

![Figure 2](image2.png)
Figure 2 The effect of treatment (SLSN, seal lice culture supernatant) and control (TCSN, tissue culture supernatant) on leucocyte migration to serum attractant represented by the migratory index (%): I, treated MØ + SLSN/fresh serum; II, treated MØ + TCSN/fresh serum; III, MØ only/fresh serum; IV, treated MØ + SLSN/PBS; V, treated MØ + TCSN/PBS; VI, treated MØ + SLSN/inactivated serum; VII, treated MØ + TCSN/inactivated serum; VIII, MØ only/PBS. 2 x 10^6 cells/100 µl were used. Time of incubation for assay was 3 hrs at 18°C. Treated MØ were prepared by incubating with the appropriate supernatant for 2 hrs. Vertical bars represent the standard error of the migratory index.

![Figure 3](image3.png)
Figure 3 Macrophage migratory responsiveness to various Ca++ ionophore stimulated host chemoattractants (CIMS). I, attractant supernatants generated with SLSN + Ca++ ionophore; II, TCSN + Ca++ ionophore; III, HBSS + Ca++ ionophore; IV, copepodid crude extract + Ca++ ionophore. 2 x 10^6 cells/100 µl were used. Treated MØ were prepared by incubating with appropriate supernatant for 2 hrs. The incubation time for the assay was 3 h. Vertical bars represent the standard error of the migratory index.

![Figure 4](image4.png)
Figure 4 Migratory activity of leucocytes with the time of incubation. Data are represented as the mean ± standard error (SE).
Immunological interaction between the fish louse and the host Atlantic salmon

The effect of sea lice secretory/excretory products or crude protein products on the macrophage (HK) migration suggests that the sea lice culture supernatant has chemotactic inhibitory characteristics, as demonstrated by the significantly lower migratory responsiveness towards the sea lice culture supernatant ($p<0.01$) (Figure 2). Of the different species of salmon experimentally infected with *L. salmonis*, the Atlantic salmon was found to be more susceptible to *L. salmonis* infection than the coho salmon (Johnson & Albright 1992a; Johnson & Albright 1992b). Hydrocortisol-implanted coho salmon also showed enhanced susceptibility to *L. salmonis* (Johnson & Albright 1992a; Johnson & Albright 1992b). These findings and the present results suggest that the chalimus secretes an immunosuppressive or anti-inflammatory chemotactic factor which seems to inhibit cell migration (Figure 5). In addition, the chemotactic inhibitory factor may be found in a soluble form in the secretory products of sea lice, which caused a significantly lower migratory responsiveness when macrophages were treated with sea lice culture supernatant alone compared to the control (TCSN) in the leucocyte migration assay (Figure 6).

Current study also assessed the migratory responsiveness of untreated and treated macrophages in Atlantic salmon serum, Ca$^{++}$ ionophore stimulated supernatant, and LPS. An enhanced migratory response to the fresh serum and a significantly lower migratory response to the heated serum were found, suggesting that a chemotactic factor (C5a) in the serum can act as a chemoattractant in this experiment and could be denatured in the heated serum (Figure 4). The C5a-like complement chemotactic factor in the serum enhances vascular permeability and promotes cellular migration, aggregation, and enzyme release of neutrophils, in addition to having the capacity to activate leukocytes and induce leukocyte chemotaxis (Griffin, 1984). The lower migratory response towards LPS exhibited by treated macrophages in response to the copepodid crude product suggests that inhibitory factors may be present in the copepodid (Figure 3).

Figure 5 shows that treated macrophages had a significantly lower migratory response to serum compared to that of untreated macrophages, possibly due to the irreversible blockage of cell receptors or by a direct suppressive affect. When the macrophages were treated with the sea lice supernatant, antigenic agents released into the supernatant were found on the macrophage receptors. In addition, cytokines such as IL-1 or similar compounds may be produced when the macrophages are treated with a sea lice secretory product, as suggested by Smith et al. (1980).

In conclusion, sea lice seem to have an immunosuppressive effect on the host immune system, triggering cell-mediated responses. Further studies also needed to identify the secretory/excretory products released by sea lice in the host as well as to identify the immune suppressive antigen found in the secretory excretory product. The findings presented here provide an insight into the mechanism of infection for the development of commercial antibiotics and vaccines against sea lice infection.

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Conflict of interest

The corresponding author declares that there is no conflict of interest.
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